GABRIEL LASMAR DOS REIS

THE INTERPLAY BETWEEN LIGHT AND TRANSCRIPTIONAL PROFILE INVOLVED IN ANTHOCYANIN BIOSYNTHESIS IN TOMATO FRUIT TISSUES

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do programa de Pós-Graduação em Fisiologia Vegetal, área de concentração em Fisiologia Vegetal, para a obtenção do título de Doutor.

Prof. Dr. Vagner Augusto Benedito
Orientador

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GABRIEL LASMAR DOS REIS

A INTERAÇÃO ENTRE A LUZ E O PERFIL TRANSCRICIONAL ENVOLVIDO NA BIOSSÍNTESE DE ANTOCIANINAS EM TECIDOS DE FRUTOS DE TOMATE

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APROVADA em 20 de outubro de 2025.
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LAVRAS – MG
2026

A minha mãe Celma, ao meu pai Josué e ao meu irmão Felipe.

Dedico

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RESUMO

As antocianinas são metabólitos vegetais especializados, conhecidos por sua pigmentação vibrante e potentes propriedades antioxidantes, oferecendo benefícios significativos tanto para a fisiologia das plantas quanto para a saúde humana. Esses compostos fenólicos hidrossolúveis aumentam a tolerância ao estresse nas plantas e desempenham um papel protetor contra doenças crônicas quando incluídos na dieta humana. A via biossintética das antocianinas é altamente conservada entre as espécies vegetais e é regulada por uma complexa rede de genes estruturais e transcricionais, sendo a luz um sinal ambiental crítico. No tomate (*Solanum lycopersicum*), uma hortícola amplamente consumida e candidata promissora para biofortificação com antocianinas, a fotoregulação da biossíntese de antocianinas ainda não é completamente compreendida. Neste estudo, utilizando linhagens de tomate enriquecidas com antocianinas (MT-*Aft/atv/hp2*), foi revelado que o acúmulo de antocianinas é restrito à casca (epicarpo) devido à limitada penetração de luz na polpa do fruto. Essa descoberta implica que a exposição à luz de forma específica por tecido e o controle transcricional, particularmente o fator de transcrição bHLH SlAN1, são determinantes. Além disso, esta pesquisa destaca o papel central da proteína COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1), uma ligase E3 de ubiquitina, na repressão da biossíntese de antocianinas em condições de escuro, ao direcionar reguladores positivos para degradação. COP1 interage com múltiplos fatores de transcrição de forma evolutivamente conservada em frutos de diversas espécies, incluindo *Arabidopsis*, maçã, pera, berinjela e tomate. Este estudo aprofunda nossa compreensão da regulação genética e pós-traducional da biossíntese de antocianinas, oferecendo *insights* valiosos para o melhoramento de culturas, aprimoramento nutricional e desenvolvimento de corantes naturais.

Palavras-chave: biossíntese de antocianinas; regulação pela luz; regulação transcricional; regulação pós-traducional.

ABSTRACT

Anthocyanins are specialized plant metabolites known for their vibrant pigmentation and potent antioxidant properties, offering significant benefits for both plant physiology and human health. These water-soluble phenolic compounds enhance stress tolerance in plants and play a protective role against chronic diseases when included in the human diet. The anthocyanin biosynthetic pathway is highly conserved across plant species and is regulated by a complex network of structural and transcriptional genes, with light serving as a critical environmental signal. In tomato (*Solanum lycopersicum*), a widely consumed horticultural crop and a promising candidate for anthocyanin biofortification, the photoregulation of anthocyanin biosynthesis remains incompletely understood. In this study, using anthocyanin-enriched tomato lines (MT-*Aft/atv/hp2*), it was revealed that anthocyanin accumulation is restricted to the peel (epicarp) due to limited light penetration into the fruit pulp. This finding implies that tissue-specific light exposure and transcriptional control, particularly the bHLH transcription factor SlAN1, are key determinants. Furthermore, the research highlights the central role of the COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) protein, a ubiquitin E3 ligase, in repressing anthocyanin biosynthesis under dark conditions by targeting positive regulators for degradation. COP1 interacts with multiple transcription factors in an evolutionarily conserved manner across fruits of various species, including *Arabidopsis*, apple, pear, eggplant, and tomato. This study deepens our understanding of the genetic and post-translational regulation of anthocyanin biosynthesis, offering valuable insights for crop improvement, nutritional enhancement, and the development of natural colorants.

Keywords: anthocyanin biosynthesis; light regulation; transcriptional regulation; post-translational regulation.

INDICADORES DE IMPACTO

Os resultados desta tese sobre a via de biossíntese de antocianinas em frutos do tomateiro representam um avanço científico com impactos significativos nos âmbitos social, tecnológico, econômico e cultural. A identificação do gene *SlAN1* como principal ativador da via de biossíntese de antocianinas, aliada à caracterização da ação repressora da proteína COP1 em condições de escuro, abre caminho para o desenvolvimento de tomates com altos teores de antocianinas, especialmente na polpa (mesocarpo), promovendo a criação de um alimento funcional de valor acessível à população. Esse enriquecimento nutricional contribui diretamente para a prevenção de doenças crônicas como diabetes tipo 2, doenças cardiovasculares e certos tipos de câncer, beneficiando potencialmente milhões de pessoas que consomem tomate regularmente. Do ponto de vista tecnológico, os resultados obtidos permitem a aplicação de estratégias genéticas em outras culturas hortícolas e frutíferas que acumulam níveis subótimos de antocianinas, ampliando a diversidade de fontes antioxidantes na alimentação. Economicamente, a pigmentação roxa do tomate representa um diferencial competitivo, agregando valor ao produto e possibilitando, além do consumo in natura, sua industrialização em molhos, extratos, sucos e ketchup com coloração atrativa, o que pode aumentar o valor agregado desses derivados. Além disso, o conhecimento gerado poderá ser aproveitado pela indústria farmacêutica para extração de antocianinas com propriedades antioxidantes e anti-inflamatórias. Culturalmente, a pesquisa estimula a conscientização sobre alimentação saudável e diversificada, promovendo mudanças positivas nos hábitos alimentares. Alinhada aos Objetivos de Desenvolvimento Sustentável (ODS) da ONU, esta pesquisa contribui diretamente para os ODS 2 (Fome Zero e Agricultura Sustentável), 3 (Saúde e Bem-Estar), 8 (Trabalho Decente e Crescimento Econômico), 9 (Indústria, Inovação e Infraestrutura) e 12 (Consumo e Produção Responsáveis), ao promover inovação científica, melhorar a qualidade nutricional dos alimentos, estimular o crescimento econômico sustentável e fomentar parcerias entre academia, setor produtivo e indústria.

IMPACT INDICATORS

The results of this thesis on the anthocyanin biosynthesis pathway in tomato fruits represent a scientific breakthrough with significant impacts across social, technological, economic, and cultural domains. The identification of the *SlAN1* gene as the main activator of the anthocyanin biosynthesis pathway, combined with the characterization of the repressive action of the COP1 protein under dark conditions, paves the way for the development of tomatoes with high anthocyanin content, especially in the pulp (mesocarp), promoting the creation of a functional food accessible to the population. This nutritional enhancement directly contributes to the prevention of chronic diseases such as type 2 diabetes, cardiovascular diseases, and certain types of cancer, potentially benefiting millions of people who regularly consume tomatoes. From a technological standpoint, the findings enable the application of genetic strategies to other horticultural and fruit crops that accumulate suboptimal levels of anthocyanins, expanding the diversity of antioxidant sources in the human diet. Economically, the purple pigmentation of tomatoes represents a competitive advantage, adding value to the product and enabling not only fresh consumption but also industrial processing into sauces, extracts, juices, and ketchup with attractive coloration, which may increase the added value of these derivatives. Furthermore, the knowledge generated could be leveraged by the pharmaceutical industry for the extraction of anthocyanins with antioxidant and anti-inflammatory properties. Culturally, the research encourages awareness of healthy and diverse eating habits, promoting positive changes in dietary behavior. Aligned with the United Nations Sustainable Development Goals (SDGs), this research directly contributes to SDG 2 (Zero Hunger and Sustainable Agriculture), SDG 3 (Good Health and Well-being), SDG 8 (Decent Work and Economic Growth), SDG 9 (Industry, Innovation and Infrastructure), and SDG 12 (Responsible Consumption and Production), by promoting scientific innovation, improving the nutritional quality of food, stimulating sustainable economic growth, and fostering partnerships between academia, the productive sector, and industry.

SUMÁRIO

FIRST PART

1 INTRODUCTION

Anthocyanins are natural pigments synthesized via the specialized metabolism of plants. Initially characterized for their role in attracting pollinators and seed dispersers, these compounds are now recognized as multifunctional, contributing to plant physiological protection. In tomato (*Solanum lycopersicum*), anthocyanin accumulation induces transcriptomic and metabolomic reprogramming consistent with shade avoidance responses, photoprotective mechanisms, and enhanced vegetative growth (CERQUEIRA *et al.*, 2022). Owing to their antioxidant capacity, anthocyanins effectively scavenge reactive oxygen species (ROS) generated under diverse stress conditions, thereby mitigating oxidative damage to plant tissues (BUER; IMIN; DJORDJEVIC, 2010; GOULD, 2004). Consequently, anthocyanins are integral to plant defense against both biotic and abiotic stressors. In addition to their physiological roles in plants, regular dietary intake of anthocyanins has been associated with human health benefits (MARTIN *et al.*, 2011).

Driven by increasing public interest in health-conscious diets, the demand for foods enriched with bioactive compounds has risen substantially (BELLIA *et al.*, 2021). Among these compounds, anthocyanins exhibit potent antioxidant and anti-inflammatory properties, contributing to the prevention of chronic diseases, including cardiovascular disorders (CASSIDY *et al.*, 2013; MAURAY *et al.*, 2010; MINK *et al.*, 2007), metabolic syndromes such as diabetes and obesity (MURAKI *et al.*, 2013; WANG, C. *et al.*, 2013), and various forms of cancer (BUTELLI *et al.*, 2008; LI, X. N. *et al.*, 2017). However, such benefits can only be achieved when a considerable amount of anthocyanins are regularly consumed in the diet.

Given the ubiquity of horticultural crops in daily diets, numerous studies have focused on elucidating the regulatory mechanisms underlying anthocyanin biosynthesis in these species (CHAVES-SILVA *et al.*, 2018). In this context, anthocyanin-enriched crops offer a practical and cost-effective strategy for improving nutritional intake, with the potential to reduce the incidence of chronic diseases through routine consumption of anthocyanin-rich produce (MARTIN *et al.*, 2011).

Among horticultural crops, tomato is the most widely consumed vegetable globally, making it an ideal candidate for anthocyanin biofortification strategies. Nevertheless, cultivated tomato varieties lack anthocyanins in their fruits due to incomplete activation of the flavonoid biosynthetic pathway in this tissue (POVERO *et al.*, 2011). Therefore, identifying and

characterizing the regulatory genes governing this pathway is essential for the development of nutritionally enhanced cultivars.

Anthocyanin biosynthesis is tightly regulated by transcription factors (TFs) responsive to developmental cues and environmental stimuli (ALBERT *et al.*, 2014). Among these, R2R3-MYB TFs function either independently or in concert with bHLH and WD-repeat (WDR) proteins, forming a MYB–bHLH–WDR (MBW) complex that orchestrates the expression of structural genes involved in anthocyanin production. These structural genes include both early biosynthetic genes (*EBGs*) and late biosynthetic genes (*LBGs*), which encode enzymes required for anthocyanin synthesis across diverse plant tissues (COLANERO; PERATA; GONZALI, 2020).

Multiple approaches have been employed to generate anthocyanin-enriched tomato lines, with several demonstrating promising outcomes (BUTELLI *et al.*, 2008; GONZALI; MAZZUCATO; PERATA, 2009; MES *et al.*, 2008; SESTARI *et al.*, 2014). Nonetheless, limitations persist in the genotypes developed to date, indicating that further refinement is necessary to optimize anthocyanin accumulation and stability.

2 LITERATURE REVIEW

2.1 Anthocyanins as multifunctional flavonoids in plant physiology and human health

Anthocyanins are specialized metabolites belonging to the flavonoid class, believed to have emerged early in plant evolution during the colonization of terrestrial environments (DAVIES; ALBERT; SCHWINN, 2012). These pigments, which display a spectrum of colors ranging from red and purple to blue and pink, were first isolated and chemically characterized by R. Willstätter (ROBINSON; ROBINSON, 1931). Structurally, anthocyanins are glycosylated polyhydroxy or polymethoxy compounds comprising two aromatic rings linked by a three-carbon bridge to a benzene ring. Although typically odorless and tasteless, their presence in edible fruits and vegetables contributes a mildly astringent flavor (MARTÍN *et al.*, 2017). To date, 702 distinct anthocyanins and 27 anthocyanidins, the aglycone forms, have been identified (KRGA; MILENKOVIC, 2019). These compounds are ubiquitous across plant species and accessions, with particularly high concentrations in some tissues of fruits and vegetables.

Throughout their life cycle, plants encounter diverse abiotic and biotic stressors, including high light intensity, heavy metal contamination, inadequate nutrient availability, salinity, drought, temperature extremes, and pathogen or insect attacks. Such conditions elevate

the production of reactive oxygen species (ROS), which can damage cellular components. To mitigate oxidative stress, plants synthesize antioxidant molecules, including anthocyanins (SHARMA *et al.*, 2019). The antioxidant activity of anthocyanins involves multiple chemical mechanisms, such as hydrogen atom donation from hydroxyl groups and metal ion chelation, enabling efficient scavenging of ROS and free radicals (GOULD, 2004). In angiosperms, anthocyanin pigmentation evolved to also serve ecological functions by attracting pollinators and seed dispersers (BUER; IMIN; DJORDJEVIC, 2010).

Beyond their physiological roles in plants, anthocyanins are recognized as bioactive compounds with potential health benefits in humans, attributed to their antioxidant and anti-inflammatory properties (LI, X. N. *et al.*, 2017; LIU, CAINI *et al.*, 2017). Diets enriched with phenolic compounds have been associated with reduced risk of chronic diseases, including cardiovascular disorders (CASSIDY *et al.*, 2013; MAURAY *et al.*, 2010; MINK *et al.*, 2007), metabolic conditions such as diabetes and obesity (MURAKI *et al.*, 2013; WANG, C. *et al.*, 2013), and some type of cancer (BUTELLI *et al.*, 2008; LI, X. N. *et al.*, 2017). Emerging evidence also suggests neuroprotective effects of anthocyanins, with promising outcomes in animal models (WANG, D. *et al.*, 2012; WANG, J. *et al.*, 2012; YOUDIM; SHUKITT-HALE; JOSEPH, 2004).

Given their multifunctional properties, anthocyanins are increasingly sought after in the food and pharmaceutical industries. Although available as dietary supplements, accumulating evidence supports the superior efficacy of consuming anthocyanins within their natural food matrix (MARTÍ; ROSELLÓ; CEBOLLA-CORNEJO, 2016). However, in most fruits and vegetables, anthocyanins are present in limited quantities and predominantly localized to the epidermal layers (BUTELLI *et al.*, 2008). Since the peel typically constitutes less than 5% of the edible mass (SESTARI *et al.*, 2014), and health benefits require consistent intake of substantial amounts, elucidating the regulatory networks governing anthocyanin biosynthesis is critical for the development of anthocyanin-enriched horticultural crops.

2.2 Tomato as a model crop for anthocyanin biofortification

Tomato (*Solanum lycopersicum* L.), a member of the Solanaceae family, is among the most globally significant and versatile horticultural crops. Wild tomato species originated in western South America, spanning regions from Ecuador through Peru to northern Chile, and extending to the Galápagos Islands (BERGOUGNOUX, 2014; KIMURA; SINHA, 2008; NOONARI *et al.*, 2015; QUINET *et al.*, 2019; VILLANUEVA, 2018). Cultivation began

during the pre-Columbian era in Central and South America. In the 16th century, the tomato was introduced to Europe as an ornamental plant, with widespread cultivation for consumption only occurring two centuries later (PERALTA; SPOONER, 2007). The domestication process led to a substantial reduction in genetic diversity, with modern cultivars retaining only ~5% of the genetic variation present in wild *Solanum* relatives (BAI; LINDHOUT, 2007). Nonetheless, interspecific hybridization remains a viable strategy for introducing novel traits through the incorporation of new genes and allelic variants.

Currently, tomatoes are cultivated on approximately 5 million hectares worldwide, yielding an annual production of ~189.1 million metric tons. Since the early 2000s, global output has increased by ~16% (FAO, 2021), with China and India leading production. Through intensive breeding programs, a diverse array of cultivars has been developed to suit a wide range of edaphoclimatic conditions and meet the requirements of various end-use applications. Tomato fruits are consumed both fresh (e.g., in salads) and processed (e.g., sauces, soups, purées, juices, and ketchup), contributing to their status as one of the most widely consumed vegetables globally (NOONARI *et al.*, 2015). Given the well-documented health benefits of anthocyanins, tomato is considered a promising candidate for biofortification strategies aimed at enhancing anthocyanin content in edible tissues.

Unlike other Solanaceae species such as eggplant (*Solanum melongena* L.) and pepper (*Capsicum annuum* L.), the cultivated tomato species usually lacks anthocyanins in its fruit due to incomplete activation of the flavonoid biosynthetic pathway in this organ (POVERO *et al.*, 2011). However, this pathway can be effectively induced through various approaches. Genetic engineering has demonstrated the feasibility of activating anthocyanin biosynthesis in tomato fruit (BUTELLI *et al.*, 2008), while conventional breeding strategies have also yielded genotypes capable of producing anthocyanin-enriched fruits (GONZALI; MAZZUCATO; PERATA, 2009; MES *et al.*, 2008; SESTARI *et al.*, 2014).

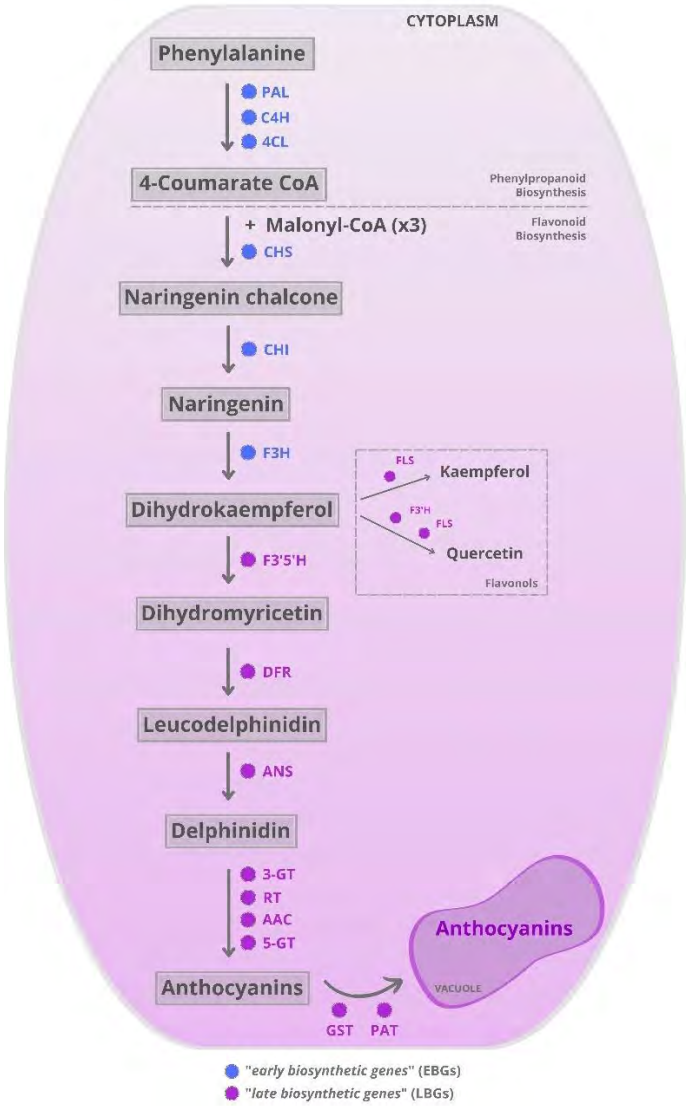
2.3 Transcriptional regulation of anthocyanin biosynthesis

The anthocyanin biosynthesis pathway is derived from the phenylpropanoid pathway, in which phenylalanine is the main precursor coming from the shikimic acid pathway (MARTENS; PREUSS; MATERN, 2010). The enzyme phenylalanine ammonia-lyase (PAL) converts the phenylalanine molecule into cinnamic acid, and this one is converted into coumaric acid by the enzyme cinnamate 4-hydroxylase (C4H). Subsequently, the enzyme 4-coumaroyl CoA ligase (4CL) converts the coumaric acid into 4-coumaroyl CoA. From the condensation of one 4-coumaroyl CoA molecule with three malonyl CoA molecules (from the fatty acid

metabolism), the naringenin chalcone is formed by the action of the chalcone synthase enzyme (CHS), which represents an important initial precursor for different subtypes of flavonoids (FERRER *et al.*, 2008). Through the subsequent action of two enzymes, chalcone isomerase (CHI) and flavanone 3-hydroxylase (F3H), the molecules naringenin and dihydrokaempferol are produced, respectively, with the dihydrokaempferol being classified as a dihydroflavonol (Figure 1). This point represents the end of the first part of the biosynthetic pathway, and the genes that encode these enzymes are named "*early biosynthetic genes*" (*EBGs*).

Subsequent reactions are mediated by "*late biosynthetic genes*" (*LBGs*). Dihydroflavonols undergo further hydroxylation by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H), which determines the hydroxylation pattern of the B-ring and influences anthocyanin coloration. The final steps involve dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose:flavonoid 3-O-glucosyltransferase (UFGT), which convert dihydroflavonols into anthocyanidins and subsequently into stable glycosylated anthocyanins (CHAVES-SILVA *et al.*, 2018). The anthocyanin biosynthesis process takes place in the cytosol, and then they are transported to the vacuole of the cells, through the action of glutathione S-transferase (GST) or putative anthocyanin transporter (PAT), where they will be stored (MARINOVA *et al.*, 2007) (Figure 1).

Figure 1 – General structural genes involved in the anthocyanin biosynthesis pathway.



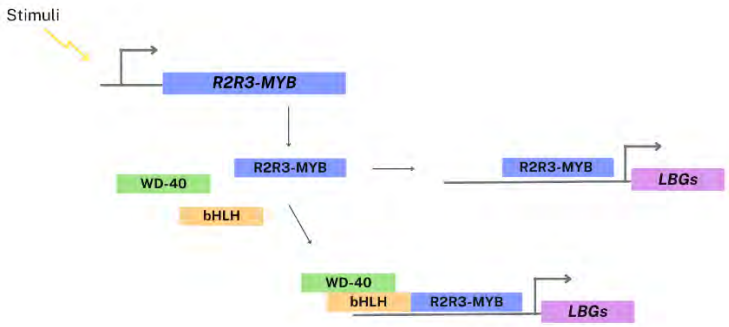
Source: By the author

The biosynthesis of anthocyanins requires the coordinated activation of structural genes, a process tightly regulated by specific transcription factors (TFs) collectively referred to as anthocyanin regulatory genes. Foundational studies in maize identified two key regulators: *Colorless1* (*C1*), encoding an R2R3-MYB transcription factor (PAZ-ARES *et al.*, 1987), and *Leaf color* (*Lc*), encoding a basic helix-loop-helix (bHLH) transcription factor (LUDWIG *et al.*, 1989). Subsequent work provided functional evidence that *C1* and *Lc* interact to form a protein complex (GOFF; CONE; CHANDLER, 1992). Further investigations revealed a third essential component: a WD40-repeat (WDR) protein, which is also required for full activation of the anthocyanin biosynthetic pathway (DE VETTEN *et al.*, 1997; WALKER *et al.*, 1999).

Research in other model plant systems, including snapdragon, petunia, and *Arabidopsis thaliana*, has identified homologous regulatory proteins involved in anthocyanin biosynthesis, underscoring the evolutionary conservation of this mechanism (BOREVITZ *et al.*, 2000; GOODRICH; CARPENTER; COEN, 1992; PAYNE; ZHANG; LLOYD, 2000; QUATTROCCHIO *et al.*, 1999; SCHWINN *et al.*, 2006; SPELT *et al.*, 2000).

It is now well established that anthocyanin biosynthesis is governed by a conserved ternary protein complex composed of R2R3-MYB, bHLH, and WDR transcription factors. This MYB–bHLH–WDR (MBW) complex is ubiquitous among angiosperms and plays a central role in activating late biosynthetic genes (LBGs) responsible for anthocyanin production in various plant tissues (KOES; VERWEIJ; QUATTROCCHIO, 2005). In certain contexts, the R2R3-MYB factor alone can initiate gene expression, although full activation typically requires the complete MBW complex (Figure 2). Intriguingly, the MBW complex also regulates the expression of negative feedback inhibitors, such as R3-MYB proteins, which suppress anthocyanin biosynthesis by competing for bHLH binding (ALBERT *et al.*, 2014; COLANERO; PERATA; GONZALI, 2018; KOES; VERWEIJ; QUATTROCCHIO, 2005).

Figure 2 – The conserved ternary complex composed of R2R3-MYB, bHLH, and WDR transcription factors to induce anthocyanin biosynthesis in plants. The wavy yellow arrow represents inductive environmental stimuli that triggers pathway activation. Black arrows indicate activation. Adapted from COLANERO *et al.* (2020).



Source: By the author

Therefore, the identification and functional characterization of species-specific regulatory genes is essential for the rational design of anthocyanin-enrichment strategies across diverse plant tissues and crop species.

2.4 Strategies for developing anthocyanin-enriched tomato fruits

Efforts to enhance flavonoid content in tomato (*Solanum lycopersicum* L.) fruits began in the early 2000s (MUIR *et al.*, 2001). Since then, multiple approaches, ranging from genetic engineering to conventional breeding, have successfully yielded anthocyanin-enriched tomato lines. Nonetheless, further refinement of existing genotypes remains possible to optimize anthocyanin accumulation and distribution.

2.4.1 Genetic engineering approaches

Initial attempts to increase anthocyanin levels in tomato fruit via genetic engineering focused on overexpressing MYB-type transcription factors. These included the endogenous *SlANT1* (MATHEWS *et al.*, 2003) and the heterologous *AtPAP1/MYB75* from *Arabidopsis thaliana* (ZULUAGA *et al.*, 2008), which led to anthocyanin accumulation in spotted regions of the fruit peel. A breakthrough was achieved by BUTELLI *et al.* (2008), who generated fully purple tomato fruits, both peel and flesh, through the fruit-specific overexpression of two regulatory genes from *Antirrhinum majus*: *Delila* (*Del, bHLH*) and *Rosea1* (*Ros1, R2R3-MYB*), driven by the *E8* promoter. This dual-gene strategy activated both early and late biosynthetic genes (*EBGs* and *LBGs*), significantly enhancing flavonoid biosynthesis.

Building on this work, ZHANG *et al.* (2015) crossed *Del/Ros1* lines with tomatoes overexpressing *AtMYB12*, a transcriptional activator of *EBGs* (LUO *et al.*, 2008). The resulting "Indigo" line exhibited elevated levels of phenylpropanoids, including chlorogenic acids, flavonols, and anthocyanins. Further enhancement was achieved by SCARANO *et al.* (2018), who introduced the *Stilbene Synthase* gene from *Vitis vinifera* into the "Indigo" background, producing the "Bronze" variety. This line accumulates three distinct polyphenol classes, flavonols, anthocyanins, and stilbenoids, and displays intense purple pigmentation in both peel and flesh.

2.4.2 Conventional breeding approaches

Conventional breeding has also enabled the development of anthocyanin-rich tomato lines. Wild tomato species such as *S. chilense*, *S. peruvianum*, and *S. lycopersicoides* can accumulate moderate anthocyanin levels under high-light conditions (BEDINGER *et al.*, 2011). Interspecific crosses between these wild species and cultivated tomato (*S. lycopersicum*) have produced accessions with anthocyanin pigmentation in fruit peels (e.g., *Anthocyanin fruit* [*Aft*] and *Aubergine* [*Abg*] lines) and vegetative tissues (e.g., *atroviolacea* [*atv*] line) (GEORGIEV,

1972; RICK; REEVES; ZOBEL, 1968). Notably, the *Aft* × *atv* cross yields the *Aft/Aft atv/atv* genotype, which exhibits pronounced purple pigmentation in the fruit peel (GONZALI; MAZZUCATO; PERATA, 2009; MES *et al.*, 2008).

- **The *Anthocyanin fruit (Aft)* allele**

The *Aft* allele originated from *S. chilense* (GEORGIEV, 1972). Under intense light, fruits from this wild species develop purple spots on the peel. *Aft* lines exhibit similar pigmentation patterns, and the genomic region responsible for this trait is located on the distal end of chromosome 10's long arm (SUN *et al.*, 2020; YAN *et al.*, 2020). Within this region, four R2R3-MYB genes have been identified: *SlANT1*, *SlANT1-like*, *SlAN2*, and *SlAN2-like*. Among them, *SlAN2-like* (*Solyc10g086290*) is the gene responsible for the *Aft* phenotype and has emerged as a key regulator of anthocyanin biosynthesis in tomato fruit.

Functional analyses revealed that the *SlAN2-like* allele in commercial *S. lycopersicum* varieties is nonfunctional due to a splicing mutation that introduces a premature stop codon, resulting in truncated proteins lacking essential R2R3 domains and the C-terminal region (COLANERO *et al.*, 2020). These truncated proteins cannot interact with bHLH partners, preventing formation of the MBW complex and anthocyanin production (Figure 3). In contrast, the *SlAN2-like* allele in *Aft* genotypes encodes a fully functional protein capable of activating anthocyanin biosynthesis via MBW complex formation (COLANERO *et al.*, 2020). Expression analyses further confirmed that *SlAN2-like* is significantly upregulated in pigmented tissues of *Aft* fruits (COLANERO *et al.*, 2020; QIU *et al.*, 2019; SUN *et al.*, 2020).

- **The *atroviolacea* (*atv*) allele**

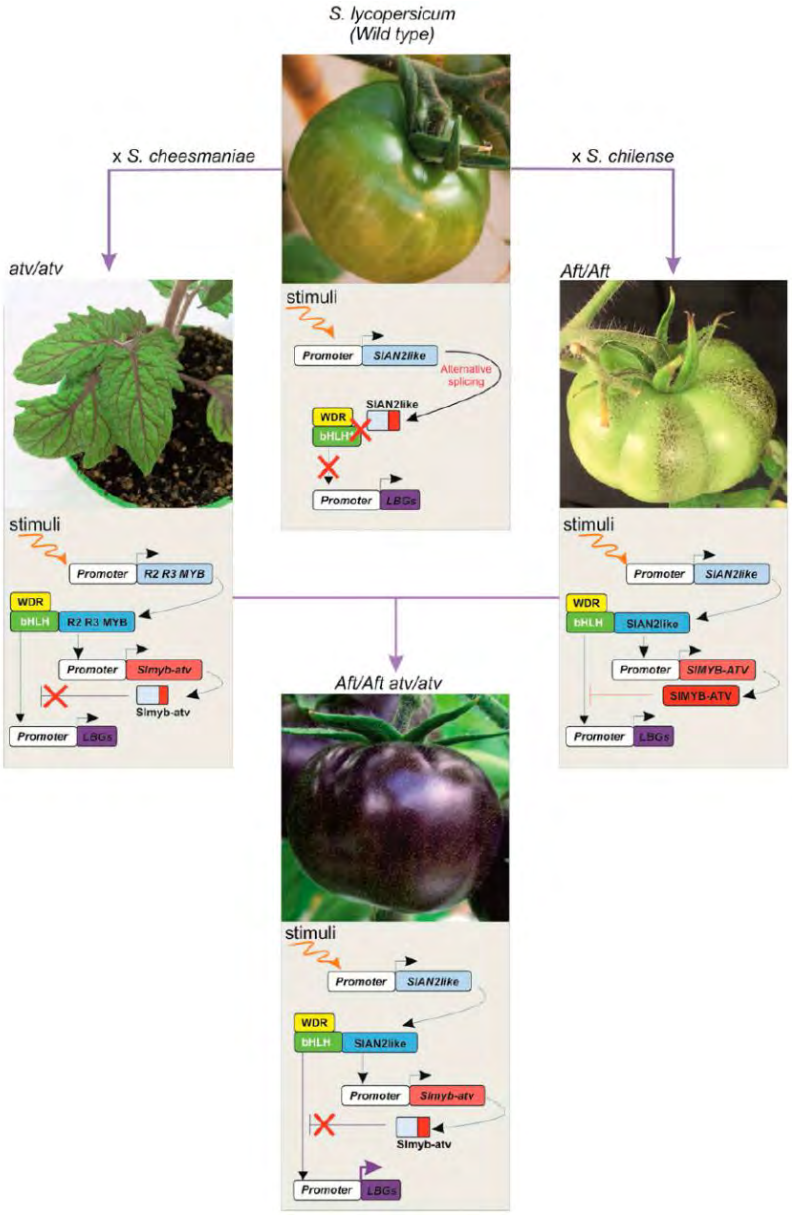
The *atv* allele originated from *S. cheesmaniae* (RICK; REEVES; ZOBEL, 1968) and is associated with elevated anthocyanin levels in vegetative tissues such as leaves, stems, and veins. The *atv* phenotype maps to chromosome 7 and is linked to the *Solyc07g052490* gene, designated *SlMYB-ATV*, which encodes an R3-MYB transcriptional repressor (CAO *et al.*, 2017; COLANERO; PERATA; GONZALI, 2018).

R3-MYB proteins possess a conserved motif in the R3 domain that facilitates interaction with bHLH factors (ZIMMERMANN *et al.*, 2004). However, lacking the domains necessary for MBW complex formation, these proteins act as competitive inhibitors by sequestering bHLH partners from R2R3-MYB activators (COLANERO; PERATA; GONZALI, 2018; SUN *et al.*, 2020). The dominant *ATV* allele encodes a functional repressor, whereas the recessive *atv*

allele contains a 4-bp insertion in exon 2, causing a frameshift and premature stop codon. This mutation yields a truncated protein devoid of the R3 domain, incapable of bHLH binding or MBW disruption (CAO *et al.*, 2017; COLANERO; PERATA; GONZALI, 2018; SUN *et al.*, 2020). Consequently, anthocyanin accumulation is enhanced in tissues of *atv* genotypes (Figure 3).

The synergic combination of the *Aft* and *atv* traits, which are correlated with an R2R3 MYB activator (*SlAN2-like*) and R3 MYB repressor (*Slmyb-atv*), respectively, leads to the perfect assembly of the MBW complex and consequently the activation of many anthocyanin structural genes (CAO *et al.*, 2017; COLANERO; PERATA; GONZALI, 2018; SUN *et al.*, 2020; YAN *et al.*, 2020) (Figure 3).

Figure 3: Breeding anthocyanin-enriched tomato lines for the production of high-anthocyanin tomato fruits. The main regulation of the anthocyanin pathway is summarized under the picture of each tomato line (COLANERO; PERATA; GONZALI, 2020).



Source: (COLANERO; PERATA; GONZALI, 2020).

- **The triple allele (*Aft/atv/hp2*) combination**

An anthocyanin-enriched tomato line was developed by stacking the *high pigment 2* (*hp2*) allele from cv. Manapal (MUSTILLI *et al.*, 1999), which confers hypersensitivity to light-mediated responses, onto the double mutant (*Aft/atv*) in the Micro-Tom background, resulting in the triple mutant MT-*Aft/atv/hp2*. This genotype exhibits a dark purple phenotype due to markedly elevated anthocyanin accumulation in the fruit peel (SESTARI *et al.*, 2014), although pigment levels remain limited in the mesocarp (GONZALI *et al.*, 2025). In addition to

anthocyanins, this line also shows increased levels of lycopene, β-carotene, and ascorbic acid without compromising yield (SESTARI *et al.*, 2014). The *hp2* allele corresponds to a loss-of-function mutation in *DEETIOLATED1* (*DET1*), a negative regulator of photomorphogenesis and anthocyanin biosynthesis that acts through COP1-mediated degradation of the transcription factor HY5 (MENCONI *et al.*, 2025). In *hp2* mutants, the absence of functional DET1 stabilizes HY5, promoting pigment accumulation even under heat stress (MENCONI *et al.*, 2025). Nevertheless, the proposed impairment of COP1 activity in *DET1*-deficient plants has only been demonstrated for HY5 and should be interpreted cautiously, as COP1 may still retain activity toward other substrates.

All the studies mentioned above represent remarkable advances in understanding the anthocyanin biosynthesis pathway in tomatoes, enabling the development of fruits enriched with this compound. However, some points and limitations must be taken into consideration. Genotypes developed through genetic engineering are classified as transgenic lines, which may be subject to strict government regulations and public misconceptions, potentially limiting their commercialization and agricultural use. On the other hand, in all genotypes developed through conventional breeding, the high anthocyanin content is restricted to the fruit peel, and in some cases, the accumulation is not uniform. Nonetheless, as demonstrated by genetic engineering studies, activation of this pathway in the fruit flesh, which constitutes the largest portion of the tomato, is achievable. Therefore, a deeper understanding of the key molecular mechanisms regulating anthocyanin biosynthesis in the different fruit tissues is critical.

3 HYPOTHESIS

SlAN1 functions as a limiting transcription factor for light-dependent anthocyanin biosynthesis in tomato fruit, and its reduced activity in the mesocarp restricts pigment accumulation.

4 OBJECTIVES

- Characterize tissue-specific anthocyanin accumulation in MT-*Aft/atv/hp2* fruits tissues (epicarp and mesocarp) under different light regimes.
- Investigate the transcriptional profile of anthocyanin-related genes in regulating anthocyanin biosynthesis in peel versus flesh under different light regimes.

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SECOND PART - ARTICLES

Artigo 1 - Redigido conforme norma do periódico científico Versão aceita para publicação	
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ARTICLE 1 – The interplay of light signaling and *SlAN1* expression in regulating anthocyanin accumulation in fruit tissues of purple tomatoes**

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Abstract

Anthocyanins are specialized plant metabolites with significant dietary value due to their anti-inflammatory properties. Research indicates that dietary intake of these phenolic compounds contributes to preventing various chronic diseases. As the most consumed vegetable worldwide, tomato (*Solanum lycopersicum*) is an excellent candidate for anthocyanin-enrichment strategies. In tomato, the activation of anthocyanin biosynthesis is light-dependent, but this

mechanism has yet to be entirely characterized. We investigated the role of light in anthocyanin biosynthesis in purple tomato fruits generated by combining the *anthocyanin fruit* (*Aft*), *atroviolacea* (*atv*), and *high-pigment 2* (*hp2*) mutations into cv. Micro-Tom (MT). MT-*Aft/atv/hp2* starts accumulating anthocyanins early during fruit development, but this accumulation is restricted to the peel (exocarp and epicarp). By manipulating light incidence in different fruit tissues, we determined that the absence of anthocyanin accumulation in the flesh results from the sun-blocking effect of the cyanic epicarp on the flesh (mesocarp), thus preventing light from penetrating deeper into the fruits. Comparative transcriptional analyses of the fruit peel and flesh indicated that the bHLH transcription factor SlAN1 (*Solyc09g065100*) may be the limiting factor for light-dependent anthocyanin accumulation in both tissues. This research enhances our comprehension of the genetic and environmental regulation of anthocyanin accumulation in fruit tissues, offering valuable insights into plant breeding for human nutrition.

Keywords: antioxidants; crop improvement; food and health; natural dye; natural genetic variation; near-isogenic line (NIL).

1. Introduction

Anthocyanins are natural pigments derived from the plant's specialized metabolism that confer red, pink, purple, or blue pigmentation to plant tissues, depending on the molecular structure and vacuolar pH for their final hue (Hichri et al., 2010; Houghton et al., 2021). Beyond the ecological notion that anthocyanins are responsible for attracting pollinators and seed dispersers, they also play a protective role due to their antioxidant activity by scavenging reactive oxygen species (ROS) that otherwise could severely damage plant tissues (Buer et al., 2010; Corso et al., 2020). Furthermore, it has been suggested that anthocyanins form a protective barrier in plant tissues by absorbing UV-B radiation potentially harmful to the photosynthetic machinery (Gould et al., 2010; Cerqueira et al., 2023). These properties make anthocyanins an important protective compound against environmental stresses. From a dietary perspective, based on their antioxidant and anti-inflammatory properties, anthocyanins are bioactive in preventing or mitigating a series of chronic diseases (Martin et al., 2011; Panchal et al., 2022), such as cardiovascular disorders (Cassidy et al., 2013), type 2 diabetes (Fallah et al., 2020), obesity (Muraki et al., 2013), and cancer (Butelli et al., 2008). Therefore, they

represent an important health-promoting compound that should be consistently incorporated into the diet.

Considering the nutritional value of horticultural crops in the human diet as sources of antioxidants, studies were carried out on these species to understand the regulatory mechanisms of the anthocyanin biosynthesis pathway (for a review, cf. Chaves-Silva et al.,, 2018). In this context, anthocyanin-enriched (cyanic) versions of fresh produce represent a critical source of anthocyanins that is readily available and cost-effective, allowing dietary enrichment simply by choosing cyanic varieties. Anthocyanins are typically found in limited quantities in cyanic products because they are often confined to the epidermal and subepidermal cells (epicarp, exocarp, or peel), which account for only 3–5% of the fruit's total mass (Sestari et al., 2014; Chaves-Silva et al., 2018). Thus, devising breeding strategies to generate anthocyanin-enriched varieties with an emphasis on the mesocarp (flesh) of horticultural crops is critical.

Worldwide, tomato (*Solanum lycopersicum*) is the most consumed vegetable. It is thus an excellent model for discovering strategies aiming at anthocyanin enrichment. Although the fruits of cultivated varieties of tomato do not accumulate anthocyanins (Povero et al., 2011; Sestari et al., 2014), some related wild species, such as *S. lycopersicoides*, *S. peruvianum*, and *S. chilense*, accumulate small amounts in the subepidermal layers under adequate light conditions (Bedinger et al., 2011; Chaves-Silva et al., 2018).

Traditional breeding has delivered some varieties with cyanic tomato fruits. The introgression of the alleles *Anthocyanin fruit* (*Aft*) and *atroviolacea* (*atv*) from *S. chilense* and *S. cheesmaniae*, respectively, into *S. lycopersicum* led to purple tomato varieties, such as the cv. Indigo Rose, with an epicarp with high levels of anthocyanins (Mes et al., 2008; Gonzali et al., 2009). The further stacking of the mutation *high pigment 2* (*hp2*) from cv. Manapal (Mustilli et al., 1999), which confers hypersensitivity to light-mediated responses, into the double mutant in the cv. Micro-Tom background led to a genotype (MT-*Aft/atv/hp2*) with very high anthocyanin content in the epicarp (Sestari et al., 2014), but low accumulation in the mesocarp (Gonzali et al., 2025). The *hp2* allele corresponds to a loss-of-function mutation in the gene *DEETIOLATED1* (*DET1*), a negative regulator of photomorphogenesis and anthocyanin biosynthesis that acts via COP1-mediated degradation of the transcription factor HY5 (Menconi et al., 2025). In *hp2* mutants, the absence of functional DET1 stabilizes HY5, promoting pigment accumulation even under heat stress conditions (Menconi et al., 2025). Nevertheless, the proposed impairment of COP1 activity in DET1 mutants has been demonstrated only for

HY5 and warrants cautious interpretation. While HY5 may evade degradation in *hp2* genotypes, COP1 may retain activity toward other substrates.

The anthocyanin biosynthesis pathway is controlled by the action of specific transcription factors (TFs), which are influenced by plant development and environmental stimuli (Albert et al., 2014). Among these, R2R3 MYBs can act individually or together with bHLH and WDR TFs in a multiprotein complex (MBW). On the other hand, R3 MYB are competitive inhibitors of the anthocyanin biosynthesis pathway mediated by the MBW. This complex controls the expression of the structural genes, the "*early biosynthetic genes*" (EBGs) and "*late biosynthetic genes*" (LBGs), which code for enzymes essential to the anthocyanin biosynthesis in different tissues (Chaves-Silva et al., 2018; Colanero et al., 2020a). In tomato, *ATV* is an R3 MYB, while *AFT* is an R2R3 MYB. This is consistent with the observation that the recessive *atv* allele, characterized by a loss-of-function due to a premature stop codon, and the dominant *Aft* allele both contribute to the enhancement of anthocyanin biosynthesis (Cao et al., 2017; Colanero et al., 2018, 2020b).

Light is one of the most critical environmental factors that control anthocyanin biosynthesis (Albert et al., 2009). Shading or dark conditions repress the expression of structural genes in this pathway (Hong et al., 2015; Liu et al., 2020). In some tomato genotypes (e.g., cv. 'Indigo Rose' and *Aft/Aft*: LA1996), the expression of anthocyanin-related genes in the epicarp starts at the early green stage and is influenced by the amount of light received on each side of the fruit, with the shaded side exhibiting a green hue compared to a darker purple hue on the side exposed to direct light (Qiu et al., 2019; Colanero et al., 2020b).

A critical unresolved question is the mechanism behind internal parenchymatic tissues being often less prone to accumulate anthocyanins than epidermal tissues in plants (Chaves-Silva et al., 2018). Here, we investigated how light influences anthocyanin accumulation in purple fruit tissues of the tomato genotype MT-*Aft/atv/hp2* by blocking light during fruit development. We explored global transcriptional activity in response to light in dissected tissues of the fruit. This work sheds light on the transcriptional regulation of anthocyanin pigmentation in response to light. Our study also provides new insights for achieving higher anthocyanin content in fleshy fruits.

2. Material and Methods

2.1 Plant material, growth conditions, and sampling

To characterize the starting point of anthocyanin pigmentation in tomato fruits, we used the cyanic genotype (MT-*Aft/atv/hp2*; Sestari et al., 2014) and the cultivar Micro-Tom (Meissner et al., 1997) as a control. The plants were grown in a greenhouse under a 16-h photoperiod with 600–700 W/m^2 radiation, 21°C ± 2°C, and 50% RH. Floral buds, flowers, and developing fruits from both genotypes were collected from the plants, placed against a black background, and photographed. The resulting images were analyzed using the NIS Elements software (Nikon Instruments).

For phenotypic characterization and RNA-seq analysis, individual flowers were covered with aluminum foil at anthesis and kept for 30 days, aiming at fruit development in the complete absence of light (Supplementary Fig. S1). Subsequently, the developed fruits were exposed to light and collected at different times: immediately (0d), 2 days (2d), and 5 days (5d) after the cover was removed. For a positive control treatment (Ctl), flowers were marked at anthesis, left exposed to normal light, and the fruits were collected after 30 days. All fruits collected were dissected into the epicarp (the peel or exocarp) and mesocarp (the flesh), and the seeds were discarded. All plant material was snap-frozen in liquid nitrogen and stored in a -80°C freezer until use. Material from three plants was collected and pooled to represent a biological replicate, and three biological replicates were used in the following analyses.

For the RT-qPCR assay, plants from MT-*Aft/atv/hp2* were grown under natural irradiation with supplemental lighting provided as needed by high-pressure sodium lighting (Hortilux 600W deep reflector HS200), with 16h photoperiod at 21°C ± 2°C. Fruits at the mature green stage of development were collected, and the peel (epicarp) and flesh (mesocarp) were dissected. The seeds were discarded. The material was collected from three individual plants, and together, they represented one biological replicate. Three biological replicates were used in the analyses. All plant material was immediately frozen in liquid nitrogen and stored in a −80 °C freezer until RNA extraction.

2.2 RNA isolation, library preparation, and sequencing

Total RNA was extracted from the epicarp and mesocarp separately using the mirVana miRNA isolation kit (Ambion, ThermoFisher) according to the manufacturer's instructions. The integrity of the RNA was visualized on a 1.2% agarose gel, and the quantity and quality were

assessed on a Nanodrop spectrophotometer. Library preparation and Illumina sequencing were performed at Novogene. Twenty-four cDNA libraries were prepared and then sequenced on an Illumina Novaseq 6000, with a configuration of 150-bp paired-end. The sequencing of transcripts revealed a total of 585.6 million reads (a mean of 23.7 million reads per library), of which 568.8 million (97%) showed sufficient quality (Q>20). The libraries were aligned on the *Solanum lycopersicum* genome assembly v.4.0 and ITAG annotation v.4.2 from SolGenomics (https://solgenomics.net; Fernandez-Pozo et al., 2015). On average, 22.2 million reads per library (94%) were uniquely mapped on the genome (Supplementary Table S1).

2.3 Pre-processing and mapping of reads

Raw FastQ data were initially submitted for quality analysis with FastQC v.0.11.5 (Andrews 2010). The cleaning step was performed with Trimmomatic v.0.39 (Bolger et al., 2014), with the following parameters: ILLUMINACLIP:TruSeq3-PE.fa:2:30:10:2:keepBothReads to remove adapters and keep both as paired reads, LEADING:3 and TRAILING:3 to remove bases below the quality of three at the start and final of each read, respectively, and MINLEN:36 to discard reads with < 36 bp. Each cleaned library was submitted for read mapping against the genome with the software Star v.2.7.5.c (Dobin et al., 2013). The counting of mapped reads for each gene was performed with featureCounts v.1.6.5 (Liao et al., 2014).

2.4 Differential expression, functional annotation, and enrichment analysis

Genes with low expression values (TPM < 1 - transcripts per million) were removed from the differential expression analysis. Differentially expressed genes were identified in R v.4.0.5 with the package DESeq2 v.1.32.0 (Love et al., 2014). All three replicates for each time point were used for the differential expression analysis, and the comparisons were performed against the same tissue. Genes with adjusted p-values < 0.05 were considered differentially expressed (DEGs). The package ggplot2 v.3.3.2 (Wickham 2016) was used to build bar charts, and ComplexHeatmap v.2.9.0 (Gu et al., 2016) for heatmaps.

Tomato gene features were retrieved from different databases. Transcription factor information was retrieved from the PlantTFDB v.5.0 (Jin et al., 2016). Annotation of genes encoding metabolic enzymes was collected from the KEGG (Kyoto Encyclopedia of Genes and

Genomes) database with GhostKOALA v.2.2 (Kanehisa et al., 2016). Gene Ontology (GO) terms were annotated with GOMAP v.1.3.4 (Wimalanathan and Lawrence-Dill 2021) to obtain a high coverage level of genome annotation. The R's match function was used to retrieve functional information from the genome and match it to a set of DEGs.

To investigate the primary functional annotations of the DEGs sets in each comparison, we conducted an enrichment analysis to identify overrepresented metabolic pathways, transcription factors, and GO terms in the dataset. This approach was applied for each DE profile, separated by up- and down-regulated genes. We considered features below the p-value < 0.05 threshold for metabolic pathways and transcription factors, and FDR < 0.05 for GO terms as overrepresented. Due to the high number of enriched GO terms, we computed the semantic similarity between terms with the mgoSim function from the R package GOSemSim v. 2.24.0 (Yu et al., 2010; Yu 2020). Following, a dimensional reduction analysis was conducted using the UMAP (Uniform Manifold Approximation and Projection) function from the UMAP v. 0.2.10 (McInnes et al., 2018) R package. dbscan function, package dbscan v. 1.1-11 (Hahsler et al., 2019) was applied to identify GO term clusters using eps of 0.4 and a minimum of five points. Ggplot2 and ggConvexHull were used to plot UMAP and bubble charts, and each clustering of GO terms was labeled by the lowest FDR value.

2.5 Transcriptional metabolic flux information analysis

The phenotypes of the cyanic mutant tomato line were investigated using an information flux deviation analysis focused on metabolic pathways related to light and anthocyanins. We reconstructed these metabolic pathways and employed a fluxomics-like approach based on gene expression and differential expression data to identify deviations in metabolic fluxes.

To conduct this analysis, we needed to reconstruct the metabolic pathways using the concept of gene orthology. This involved retrieving information from KEGG orthology (Kanehisa et al., 2016), identifying orthologs from Arabidopsis, using OMA (Altenhoff et al., 2024), and utilizing metabolic genes previously identified in tomato.

2.6 RNA isolation, DNase treatment, and cDNA synthesis

Total RNA was extracted from epicarp and mesocarp separately using Trizol reagent (Invitrogen). The integrity of the RNA was visualized on a 0.8% agarose gel, and the quantity

and quality (ratio 260/280 and 260/230 between 1.8 and 2.2) were measured on a Nanovue spectrophotometer. RNA samples (5.0 µg) were treated with a Turbo DNA-free kit (Life Technologies) to eliminate residual genomic DNA. Subsequently, 1 µg of the treated RNA from each sample was submitted to cDNA synthesis using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher).

2.7 Real-time PCR analysis

For the quantitative real-time PCR (RT-qPCR), the standard QuantiNova SYBR Green PCR Kit (QIAGEN) protocol was used. The compounds of these reactions were 1.5 µl cDNA, 1.5 µl of each primer (forward and reverse) at a final concentration of 1 µM, 7.5 µl of QuantiNova SYBR Green PCR Master Mix (QIAGEN), and 3.0 µl of water to a final reaction volume of 15 µl, for each reaction. These final reactions were incubated at 95 °C for 2 minutes, followed by 40 cycles at 95 °C for 5 seconds and 60 °C for 10 seconds. Then, the samples were heated from 55 to 95 °C with an increase of 1 °C to acquire the melting curve of the amplified products. All reactions were run in duplicates.

The efficiency of each primer was calculated by a standard curve of a 1:5 serial dilution of a cDNA pool. Primer sequences are listed in Supplementary Table S2. The normalized comparative Cq (quantitative Cycle) method was used (Pfaffl 2001) to calculate relative expression, using *Solanum U6* and *5.8S* as reference genes.

2.8 Statistics

Statistical analyses of the RT-qPCR data were performed using the R software (Team 2013). The normality of variables was assessed using the Shapiro-Wilk test. The T-test was applied to the data. All tests were used with a significance level of 95% ($P \leq 0.05$).

3. Results

3.1 The onset of anthocyanin pigmentation in the cyanic tomato fruit (MT-*Aft/atv/hp2*)

We monitored flowering and fruit development in MT-*Aft/atv/hp2* plants and identified that anthocyanin accumulation starts right after flower senescence. As the petals fell off and the

young fruit was directly exposed to light, anthocyanin accumulated in the epicarp (Fig. 1A). In contrast, fruits of the control genotype (cv. Micro-Tom) did not show visible anthocyanin pigmentation, as expected (Fig. 1B). This pattern of anthocyanin accumulation in MT-*Aft/atv/hp2* plants is independent of further fruit development but restricted mainly to the epicarp, while the mesocarp and the region under the sepals remained acyanic (Fig. 1C).

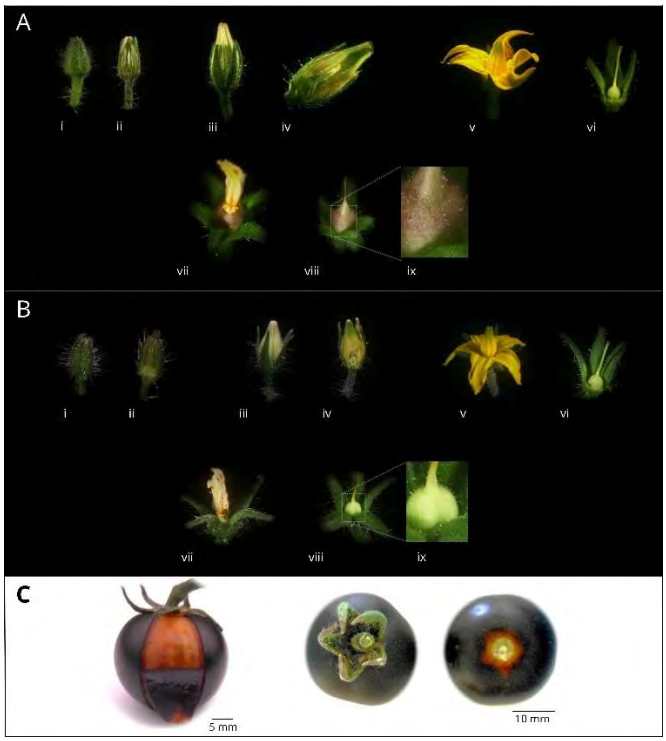

Fig. 1. Monitoring the flowering and fruit development of two Micro-Tom (MT) genotypes and light-dependent anthocyanin accumulation patterns in purple tomato fruit.

(A) Floral buds, flower, and developing fruit in the purple-fruit genotype, MT-*Aft/atv/hp2*, and **(B)** the regular, red-fruit cv. Micro-Tom (control). i, Developing floral bud; ii, Cross-section of the developing floral bud; iii, Immature flower; iv, Cross-section of the immature flower; v, Flower anthesis; vi, Flower anthesis without the petals; vii, Floral senescence; viii, Fruit in early development at floral senescence; ix, Zoom in on the early developing fruit shown in viii. **(C)** Lack of anthocyanin accumulation in the mesocarp and proximal region of mature fruits (MT- *Aft/atv/hp2*) when growing under normal light conditions. Notice the lack of anthocyanin accumulation in the epidermis under the calyx due to the lack of direct light exposure.

3.2 Light activates anthocyanin biosynthesis in the tomato mesocarp

Based on the light-dependent pattern of anthocyanin pigmentation in MT-*Aft/atv/hp2* purple fruits, we investigated the metabolic response when restricting light incidence on the fruit from its first developmental stages and then exposing the mature green fruit to light. Immediately after anthesis, individual flowers were covered with aluminum foil to allow the

fruit to develop for 30 days to the mature green stage in the complete absence of light (Supplementary Fig. S1). As a control, flowers from other plants were marked simultaneously but not covered. Compared to control fruits, which developed a dark purple phenotype in the epicarp and a green phenotype in the mesocarp (Fig. 2), the covered fruits remained acyanic at the removal of the cover (0d; Fig. 2). Notably, even without light, fruits developed normally in size and shape, reaching a diameter of 3 cm.

To investigate the activation of the anthocyanin biosynthesis after the fruit was fully developed in the dark, we exposed them to normal light conditions for up to five days after the cover had been removed and examined the anthocyanin pigmentation phenotype in the epicarp and mesocarp. Exposure to light for two days (2d) showed visible signs of anthocyanin accumulation in sectors of the fruit epicarp and mesocarp (Fig. 2). Furthermore, exposure to light for five days (5d) led to a notable increase in purple pigmentation in the entire epicarp and, surprisingly, also in the mesocarp (Fig. 2). The progressive development of the purple hue in the fruits is shown in Supplementary Fig. S2.

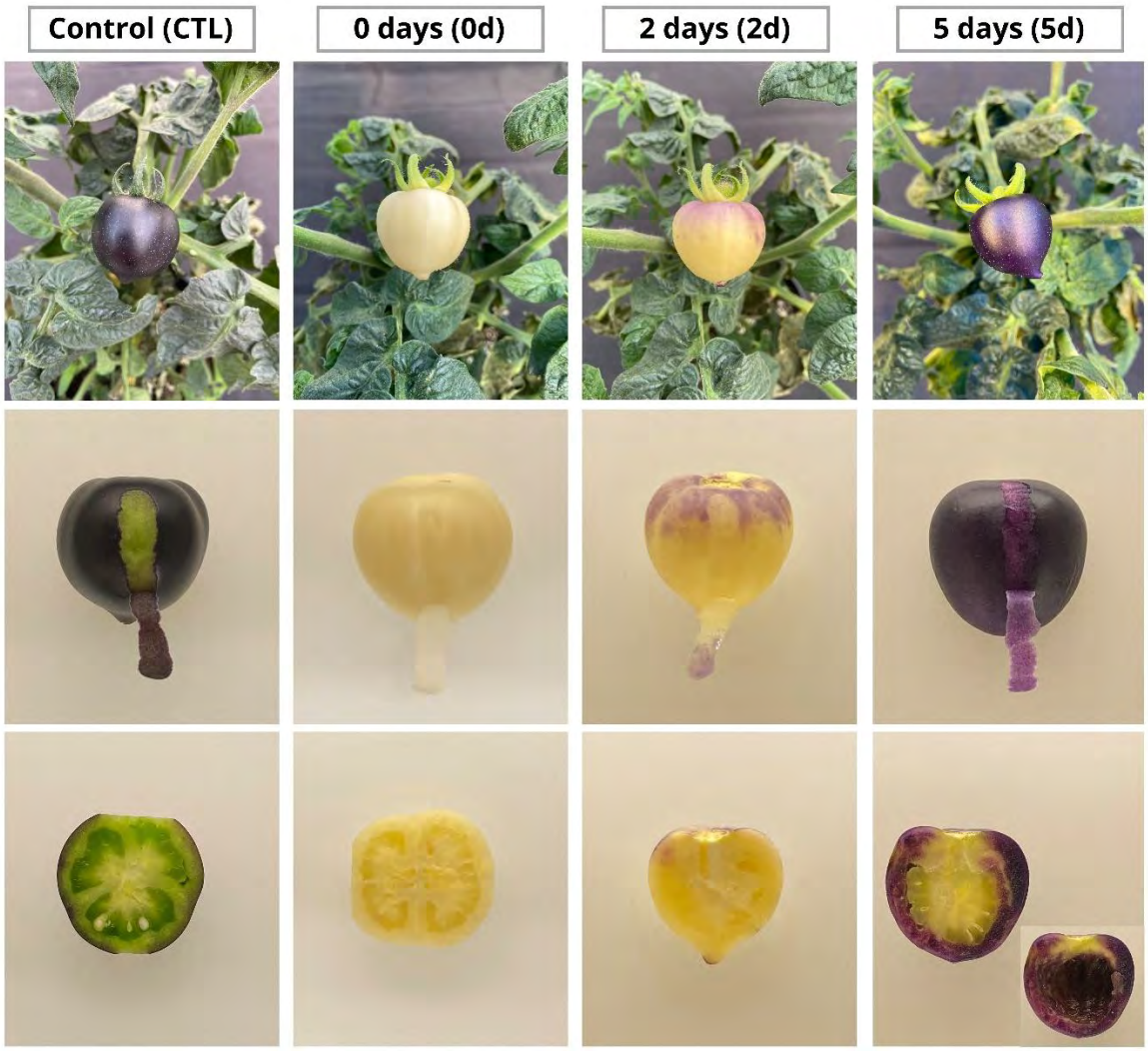

Fig. 2. Phenotypic characterization of the anthocyanin pigmentation pattern.

The epicarp and mesocarp of the cyanic tomato genotype (MT- *Aft/atv/hp2*) developed in the dark for 30 days post-anthesis. Tissues of fruit developed under different light conditions: not covered (control); immediately after the removal of the foil cover (0d); 2 days (2d); and after 5 days (5d) after cover removal. The 2d and 5d fruits were cut longitudinally to better visualize the anthocyanin accumulation in the mesocarp tissue. The inset of the 5d mesocarp cross-section displays the internal side of the mesocarp by removing the inner fruit tissues.

3.3 Global transcriptional expression analysis of tomato fruit tissues in response to light

To further understand the molecular mechanisms behind the light-mediated transcriptional regulation of anthocyanin accumulation in the tomato fruits, we carried out RNA-seq analysis of the epicarp and mesocarp of the treatments shown in Fig. 2. By comparing the epicarp of control (fruits developed under light) and fruits exposed to light for 0 (immediately after uncovering), 2, and 5 days after uncovering, we identified 4559, 5859, and 3900 DEGs, respectively. The expression of 983 genes in the epicarp differed in 0, 2, and 5 days of light exposure compared to the control. Meanwhile, we found 1722 (0d), 5705 (2d), and 4937 (5d) DEGs in the mesocarp compared to the control (Fig. 3A; Supplementary Table

S3). In this tissue, 549 genes were identified as differentially expressed coincidently in 0, 2, and 5 days of light exposure, compared to the control (Fig. 3B). The heatmap representing all DEGs is shown in Supplementary Fig. S4. Expression values for TPM and DEGs can be accessed in the Supplementary Table S4 and S5, respectively. This analysis revealed a light-triggered transcriptional network involved in anthocyanin accumulation in fruit tissues.

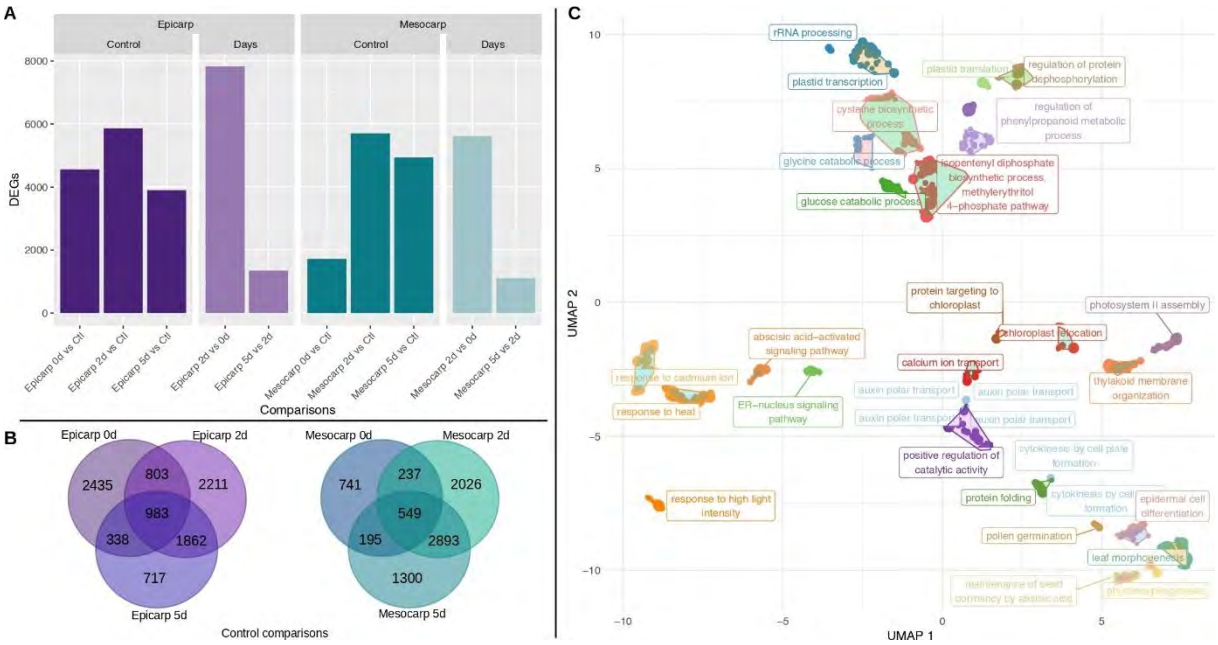

Fig. 3. Differentially expressed genes (DEGs) and enriched GO terms in tomato fruit tissues (MT-*Aft/atv/hp2*) in response to different light exposure conditions.

(A) Total number of DEGs in different comparisons. **(B)** Venn diagrams for differential expression within the same tissue in different light conditions versus the control (fruit grown under normal light conditions). **(C)** Summary of all enriched GO terms (biological process) clustered by semantic similarity using UMAP (Uniform Manifold Approximation and Projection). Each cluster was labeled by the GO term with the lowest FDR. d, days; Ctl, control (fruit grown under light conditions); Log2FC, logarithmic base 2 of fold change.

Enrichment analyses of functional gene annotations can reveal critical transcriptional changes associated with a phenotype. Therefore, we conducted enrichment analyses of metabolic pathways, transcription factor (TF) families, and gene ontology (GO) terms to identify distinct patterns triggered by light in fruit tissues. Seventy-five metabolic pathways were enriched in both the epicarp and mesocarp upon light exposure, including those directly or indirectly associated with anthocyanin biosynthesis and specialized metabolism: flavone and flavonol biosynthesis (map00944); flavonoid biosynthesis (map00941); phenylalanine metabolism (map00360); phenylalanine, tyrosine, and tryptophan biosynthesis (aromatic amino acids: map00400); along with the biosynthesis of carotenoids (map00906) and terpenes

(map00900), and the degradation of geraniol (map00281). Aromatic amino acid biosynthesis was not enriched in the mesocarp at 0d light exposure but at 2d and 5d of light exposure when compared with the mesocarp of the control fruit developed under light conditions (Supplementary Fig. S5; Supplementary Table S6).

Transcription factors (TFs) play critical roles in modulating gene expression. We identified 15 TF families enriched in the various DEG comparisons (Supplementary Fig. S5). The most noticeable family was MYB at 2d and 5d light exposure compared to the control. Other anthocyanin-related TF families were identified, such as SQUAMOSA promoter-binding protein-like (SBP box) and Double B-box (DBB) proteins (Supplementary Fig. S5). Further analysis of the DEG sets allowed us to identify 27 clusters of enriched GO terms associated with light-mediated responses. A clustering of light responses was found with 20 GO terms, which include response to far-red light (GO:0010218), response to red light (GO:0010114), response to high light intensity (GO:0009644), and the profiles with the highest number of enriched GO terms were downregulated in the mesocarp at 2d and 5d (against control). Interestingly, anthocyanin-containing compound biosynthesis (GO:0009718) was enriched in both tissues (Supplementary Fig. S5) when anthocyanin accumulation became perceptible.

3.4 Expression of photoreceptor genes in the anthocyanin biosynthesis pathway

We propose a model for suppressing anthocyanin accumulation in the MT-*Aft/atv/hp2* mesocarp, where the pigmented epicarp creates a shading effect on the internal tissues of the fruit, thereby preventing the mesocarp from initiating anthocyanin biosynthesis. To evaluate this hypothesis, we examined the transcriptional level of photoreceptor genes: phytochromes (sensors of red and far-red light: *SlPHYA*, *SlPHYB1*, and *SlPHYB2*), cryptochromes (sensors of blue/UV light: *SlCRY1a*, *SlCRY1b*, *SlCRY2*, and *SlCRY3*), and *SlUVR8* (a sensor of UV-B light). In our study, *SlCRY3* (*Solyc08g074270*) showed a higher expression in the epicarp than the mesocarp of fruits developed under normal light conditions (Fig. 4A; Supplementary Table S7). Furthermore, *SlCRY3* expression was repressed in the epicarp of the fruit just uncovered (0d) compared to the control. In contrast, no difference was observed between the mesocarp at 0d and the control fruit (Fig. 4A; Supplementary Table S7). *SlCRY3* was upregulated in the mesocarp at 2d of light exposure compared to 0d, indicating that light reached the mesocarp and activated its expression at 2d in this tissue. On the other hand, in neither tissue, *SlCRY1a* (*Solyc04g074180*), *SlCRY1b* (*Solyc12g057040*), or *SlCRY2* (*Solyc09g090100*) showed differential expression between the epicarp and mesocarp of fruits developed under light

(control), or when comparing the same tissue at 0d with the control conditions (Fig. 4A; Supplementary Table S7).

The UV-B-responsive photoreceptor gene *SlUVR8* (*Solyc05g018630*) showed higher expression in the epicarp compared to the mesocarp in the control conditions. Furthermore, it was upregulated in the mesocarp at 2d and 5d light exposure compared to the control mesocarp (Fig. 4A; Supplementary Table S7). The expression of genes coding for the phytochromes *SlPHYB1* (*Solyc01g059870*) and *SlPHYB2* (*Solyc05g053410*) was repressed in the epicarp control compared to the mesocarp control. In contrast, *SlPHYA* (*Solyc10g044670*) did not show a significant difference in this comparison (Fig. 4A; Supplementary Table S7).

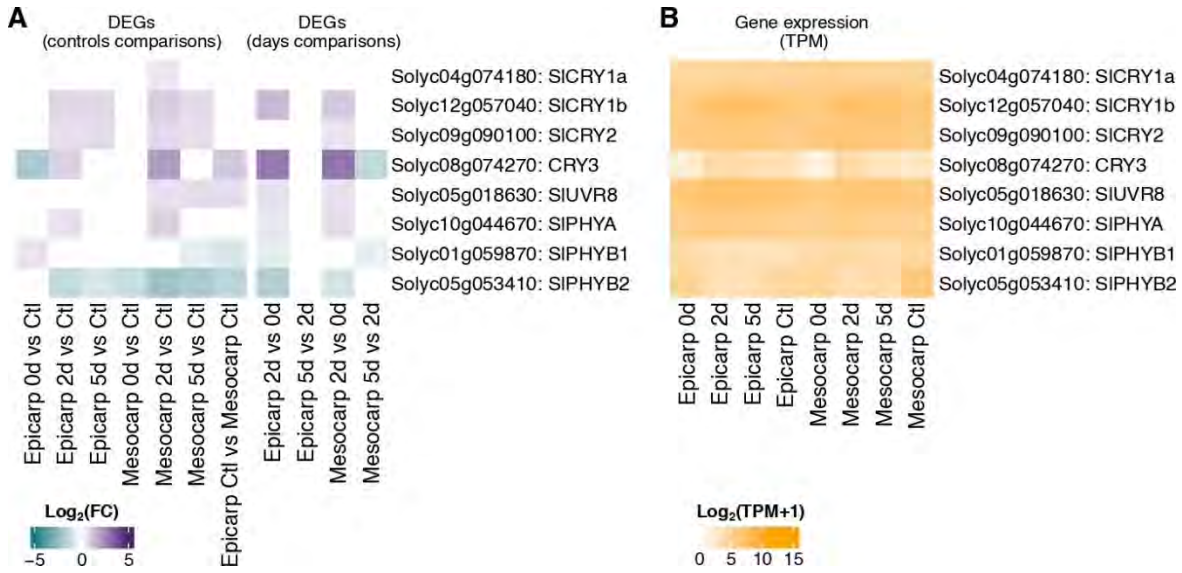

Fig. 4. Transcriptional level of the anthocyanin photoreceptor genes in tissues of tomato fruits (MT-*Aft/atv/hp2*).

(A) Differential expression (log2 FC) of the photoreceptor genes involved in the anthocyanin biosynthesis pathway. **(B)** Expression [log2 (TPM+1)]. Abbreviations: Ctl, control; d, days of light exposure after cover removal; DEGs, differentially expressed genes; Log2FC, base-2 logarithm of fold change; TPM, transcripts per million.

3.5 Analysis of anthocyanin-related transcription factor gene expression patterns in MT-*Aft/atv/hp2* tomato fruit tissues

We analyzed the expression of some transcription factors that act upstream of the MBW complex in anthocyanin biosynthesis activation (Fig. 5A; Supplementary Table S8). The expression of the *SlHY5* (*Solyc08g061130*) gene was upregulated in the epicarp at 0d light exposure and in the mesocarp at 0d and 2d compared to the control tissues of fruits developed under normal light conditions. Interestingly, *SlHY5* expression was induced in the acyanic epicarp developed without light (0d) compared to the cyanic epicarp (control). The transcription

factor *SlWRKY* (*Solyc10g084380*) was repressed in the epicarp at 0d light exposure compared to the cyanic fruit control. In contrast, it was induced in the mesocarp at 2d and 5d light exposure compared to the acyanic mesocarp of the control fruits. Furthermore, *SlWRKY* was induced in the epicarp compared to the mesocarp in control conditions. This expression pattern aligns with the development of anthocyanin pigmentation observed in the MT-*Aft/atv/hp2* fruit tissues. We also observed that the transcriptional levels of *CONSTITUTIVE PHOTOMORPHOGENIC 1* (*COP1 homolog* [*Solyc12g005950*] and *COP1-like isoform X1* [*Solycg11g011980*]) were similar across the analyzed conditions, except for the epicarp and mesocarp at 0d, where the *COP1-like isoform X1* was expressed at lower levels (Fig. 5A; Supplementary Table S8).

Among the MYB TF genes, *SlANT1* (*Solyc10g086260*) and *SlANT1-like* (*Solyc10g086270*) were not expressed in any of the tissues or treatments analyzed, except for the epicarp at 0d, where *SlANT1-like* showed a very low value (Fig. 5A; Supplementary Table S8). The *SlAN2* (*Solyc10g086250*) showed very few transcripts in the epicarp and mesocarp in all conditions analyzed. By contrast, *SlAN2-like* (*Solyc10g086290*) showed high expression levels in all tissues and conditions analyzed. In the epicarp, there was no differential expression of the *SlAN2-like* at 2d and 5d light exposure, while it was downregulated at 0d compared to the light-exposed control. *SlAN2-like* expression was induced at 2d and 5d in the mesocarp but was not differentially expressed at 0d compared to the control (Fig. 5A and B; Supplementary Table S8). Furthermore, the RT-qPCR confirmed the slight difference between the *SlAN2-like* expression in the anthocyanin-pigmented epicarp and the non-pigmented mesocarp (Fig. 5C and D).

SlAN2-like interacts with the constituent factors bHLH1 (SlJAF13) and WDR (SlAN11) to form the first MBW complex (Chaves-Silva et al., 2018). In our study, *SlJAF13* (*Solyc08g081140*) and *SlAN11* (*Solyc03g097340*) were expressed in all cyanic and acyanic tissues analyzed (Fig. 5A; Supplementary Table S8). In the epicarp, *SlJAF13* expression was induced at 2d light exposure, whereas *SlAN11* expression was lower at 0d but higher at 2d and 5d when compared to the light-exposed control. In the mesocarp, *SlJAF13* and *SlAN11* expression levels were not different at 0d compared to the control, but they were induced at 2d and 5d. Subsequently, the formation of the first MBW complex induces the expression of *bHLH2* (*SlAN1*: *Solyc09g065100*). This, in turn, replaces bHLH1 and leads to the assembly of the second MBW complex, ultimately activating the anthocyanin structural genes. In both tissues, *SlAN1* expression was minimal at 0d light exposure, whereas it highly increased at 2d and 5d (Fig. 5B; Supplementary Table S8), which correlates with anthocyanin pigmentation in tomato fruit tissues.

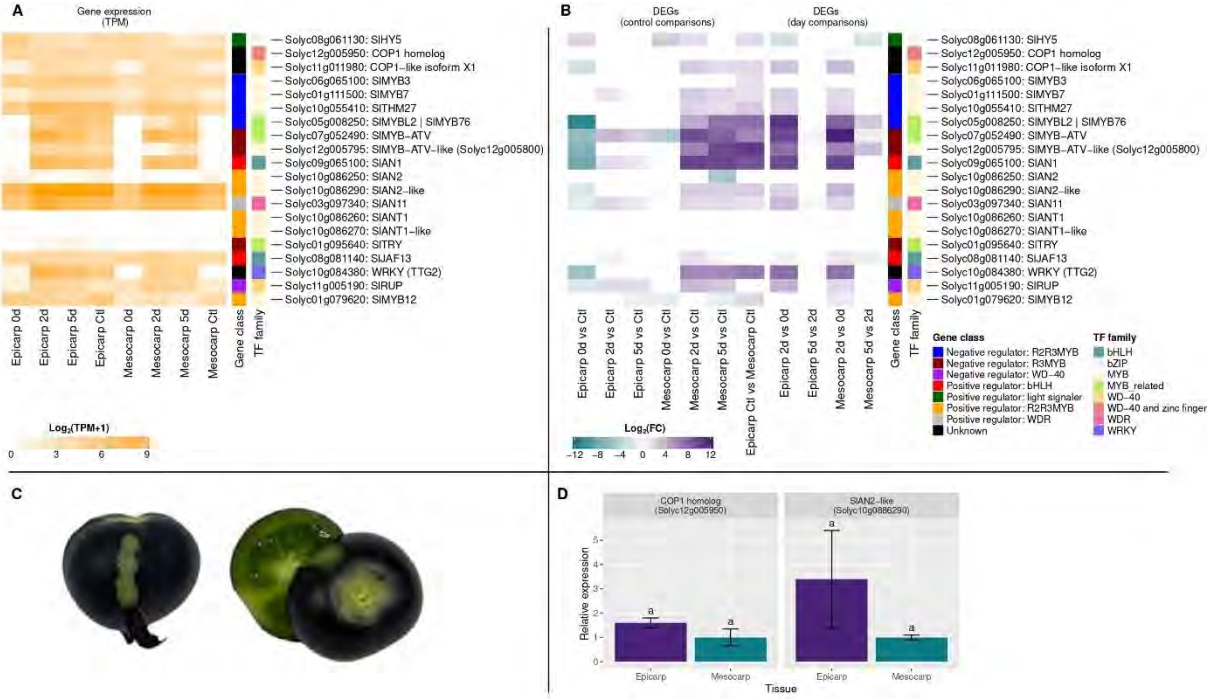

Fig. 5 Transcriptional level of the anthocyanin biosynthetic regulatory genes in tissues of tomato fruit (MT-*Aft/atv/hp2*).

(A) Expression [log2 (TPM+1)] and **(B)** differential expression (log2FC) of the anthocyanin biosynthetic regulatory genes in the epicarp and mesocarp of tomato fruits (MT-*Aft/atv/hp2*) exposed to different light conditions. **(C)** MT-*Aft/atv/hp2* at the mature green stage of development. **(D)** RT-qPCR analysis of *SlAN2-like* (*Solyc10g086290*) and *COP1 homolog* (*Solyc12g005950*) genes was performed in fruit tissues (epicarp and mesocarp) of MT-*Aft/atv/hp2* at the mature green stage of development. The fruits were developed under normal light conditions. Data is means of three biological replicates. The same letters indicate no significant differences at $P \leq 0.05$. Abbreviations: Ctl, control; d, days of light exposure; DEGs, differentially expressed genes; Log2FC, base-2 logarithm of fold change, TPM, transcripts per million.

3.6 Expression of specific MYB regulators of anthocyanin biosynthesis in MT-*Aft/atv/hp2* tomato fruit tissues

We examined the role of the second MBW complex in activating the expression of the negative anthocyanin regulators: *SlMYB-ATV* (*Solyc07g052490*), *SlMYB-ATV-like* (*Solyc12g005795*), *SlTRY* (*Solyc01g095640*), *SlMYB3* (*Solyc06g065100*), *SlMYB7* (*Solyc01g111500*), *SlMYB32* (*THM27* - *Solyc10g055410*), and *SlMYBL2/SlMYB76* (*Solyc05g008250*). Although these MYB repressors were expressed in all samples (Fig. 5A; Supplementary Table S8), the *SlMYB-ATV* expression pattern matches that of *SlAN1*, suggesting that the second MBW complex coordinates the transcription of both genes.

3.7 Expression of structural genes associated with anthocyanin biosynthesis in purple fruits

Regulatory and structural genes regulate the anthocyanin biosynthesis pathway (Fig. 6A). In the epicarp, the expression of almost all structural genes was highly downregulated at 0d of light exposure, compared with the control. In contrast, at 2d and 5d of light exposure, their expression levels were similar to the control (Fig. 6B; Supplementary Table S9). Interestingly, in the mesocarp at 2d and 5d light exposure conditions, EBGs and LBGs were highly upregulated compared with the acyanic mesocarp control developed under normal light conditions, matching the anthocyanin accumulation in the fruit tissues (Fig. 6B; Supplementary Table S9).

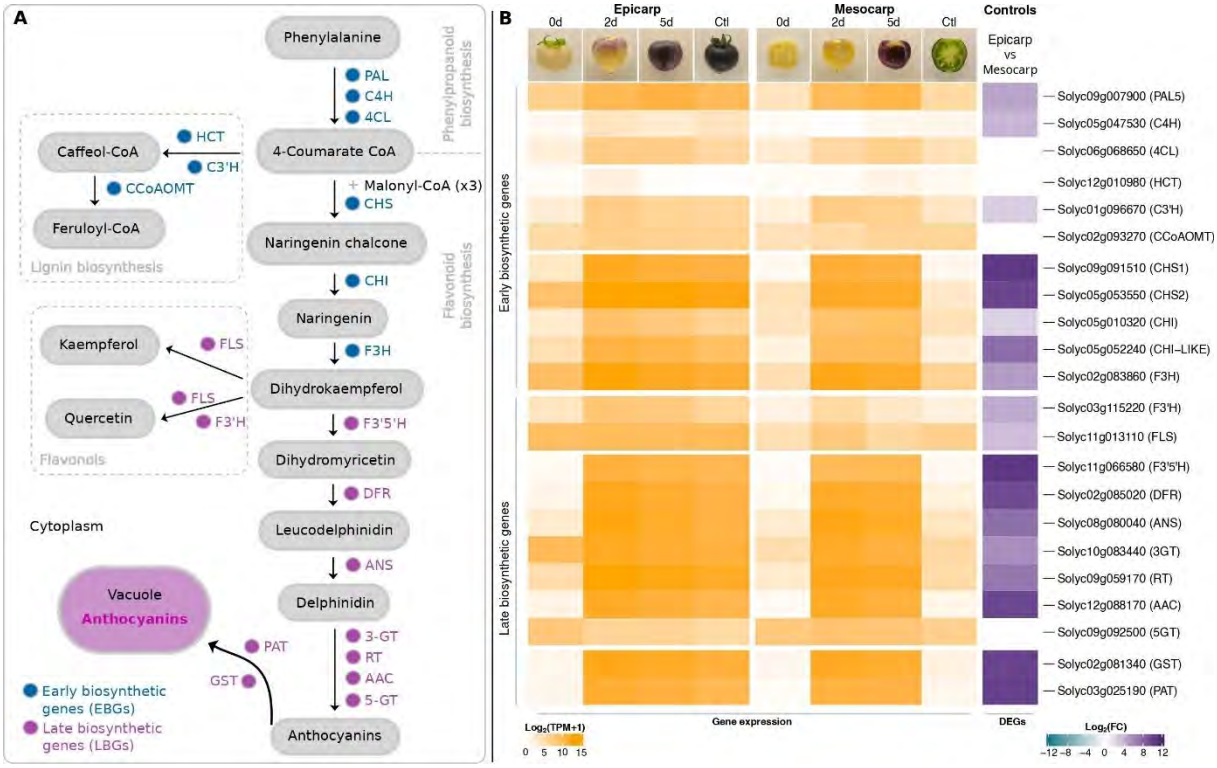

Fig. 6. Anthocyanin biosynthesis pathway and expression patterns of the early biosynthetic genes and late biosynthetic genes in tissues of tomato fruits (MT-*Aft/atv/hp2*).

(A) Anthocyanin biosynthesis pathway Adapted from Qiu et al. (2019). **(B)** Expression [log10 (TPM+1)] and differential gene expression (log2 FC) of the anthocyanin Early biosynthetic genes and Late biosynthetic genes in the epicarp and mesocarp of purple tomato fruits (MT-*Aft/atv/hp2*). d, days; Ctl, control; TPM, transcripts per million reads; Log2 FC, base-2 logarithm of fold change.

4. Discussion

4.1 Light-dependent anthocyanin biosynthesis activation in the MT-*Aft/atv/hp2* fruits

Genetic and environmental parameters directly influence the anthocyanin biosynthesis pathway in the tomato fruit (Albert et al., 2014). Light-mediated signals are usually essential to activating this pathway in different tissues (Liu et al., 2018). In addition, fruit development also influences anthocyanin accumulation, with some anthocyanin-enriched tomato genotypes only starting to accumulate this compound after a specific stage (Qiu et al., 2019; Sun et al., 2020). Our cyanic genotype MT-*Aft/atv/hp2* is an anthocyanin-enriched tomato line that accumulates anthocyanins in the subepidermal layer of the epicarp (the peel), thus developing dark purple fruits. This line was developed by introgressing natural genetic variation from two wild species and a cultivar of tomato (the loci *Aft*, *atv*, and *hp2*) to create a near-isogenic line (NIL) in the cv. Micro-Tom background: the MT-*Aft/atv/hp2* (Sestari et al., 2014).

Fruits of tomato lines containing both alleles *Aft* and *atv* in homozygosity in cv. Indigo Rose showed progressive accumulation of anthocyanins, starting right before the mature green stage on the side directly exposed to light, whereas the shaded side remained green at this stage (Qiu et al., 2019; Sun et al., 2020). On the other hand, MT-*Aft/atv/hp2* fruits started accumulating anthocyanins in the epicarp right after petal senescence (Fig. 1A). The difference in pigmentation pattern is due to the *hp2* allele, a loss of *DEETIOLATED* (*DET1*) function, a negative regulator of light signal transduction, conferring hypersensitivity to light (Levin et al., 2003). However, when growing under regular light exposure, anthocyanin accumulation in MT-*Aft/atv/hp2* fruits remained restricted to the epicarp. In contrast, the mesocarp and the region under the sepals remained acyanic (Fig. 1C). This pattern shows that light is essential to activating anthocyanin biosynthesis in the purple tomato fruits.

4.2 Anthocyanin pigmentation patterns correlate with light exposure

Anthocyanin accumulation is commonly restricted to the subepidermal cells and absent in the parenchymal cells of the mesocarp, mesophyll, and cortex. Substrate is likely to be available in these cells since the expression of specific transgenic MYB and bHLH transcription factors leads to high anthocyanin accumulation in the inner tissues of the tomato fruits (Butelli et al., 2008; Cerqueira et al., 2023). Therefore, other mechanisms should explain this "parenchymal recalcitrance", a widespread phenomenon throughout angiosperms (Chaves-Silva et al., 2018).

Here, we demonstrated that the restriction of light incidence over MT-*Aft/atv/hp2* fruits completely inhibited the anthocyanin pigmentation in all tissues (Fig. 2). Similar results of the

anthocyanin pigmentation inhibition in the dark were observed in tomato (Xu et al., 2022), apple (Li et al., 2012), broccoli (Liu et al., 2020), chrysanthemum (Hong et al., 2015), and eggplant (Jiang et al., 2016; Li et al., 2024), confirming that light is a major factor controlling anthocyanin biosynthesis (Liu et al., 2018). Subsequently, five days of light exposure of the non-pigmented, mature green MT-*Aft/atv/hp2* fruits, grown in the dark for 30 days to light conditions, led to rapid activation of the anthocyanin biosynthesis in both the epicarp and mesocarp (Fig. 2). This observation shows that the activation of the anthocyanin biosynthesis pathway in the MT-*Aft/atv/hp2* fruit depends directly on the incidence of light and is developmentally independent. Moreover, although some studies have successfully obtained anthocyanin accumulation in the mesocarp via transgenic methods (Butelli et al., 2008; Sun et al., 2020), our study is the first to report anthocyanin pigmentation in the tomato mesocarp through natural genetic variation. These findings led us to reason that the pigmented epicarp of MT-*Aft/atv/hp2* acted as a light-blocking layer starting in the first stage of fruit development, thus preventing light from reaching the mesocarp. Our experimental design, therefore, allowed the inhibition of the early pigmentation of the epicarp and facilitated the penetration of light into the non-pigmented epicarp, triggering anthocyanin biosynthesis in the mesocarp (Supplementary Fig. S3).

4.3 Photoreceptors involved in the anthocyanin biosynthesis activation in tomato fruits

Plants use specific photoreceptor classes to receive light signals and coordinate stimulus responses. The cryptochrome *CRY3* participates in anthocyanin biosynthesis in eggplant, purple broccoli, and petunia. *CRY3* expression is repressed during shading conditions and highly induced by light (Li et al., 2017; Fu et al., 2020; Liu et al., 2020). In petunia, while *CRY3* was repressed by exposure to red light compared to white and blue lights, *CRY1* and *CRY2* did not show significant changes in response to the light quality (Fu et al., 2020). The synergistic effect of blue and UV-B light promoted anthocyanin accumulation in the epicarp of the tomato *Aft* line (Kim et al., 2021). In our study, *SlCRY3* and *SlUVR8* were the only photoreceptors transcriptionally induced in the cyanic epicarp compared with the acyanic mesocarp in control conditions. *SlUVR8* expression increased in both tissues along the days of light exposure for the fruits grown in the dark, whereas *SlCRY3* peaked at 2d when anthocyanin accumulation started becoming noticeable (Fig. 4B). Based on these gene expression patterns and the current understanding that short-wavelength radiation (*i.e.* blue and UV lights) promotes anthocyanin

accumulation, we infer that *SlCRY3* and *SlUVR8* are the primary photoreceptors activating the anthocyanin pigmentation in tomato fruits.

Therefore, the anthocyanin pigmentation of the MT-*Aft/atv/hp2* epicarp starts at the first stage of fruit development and directly influences the quantity and quality of light in the mesocarp by blocking short-wavelength radiation. This light-blocking effect leads to the inactivation of light-induced signal transduction in the mesocarp, which is necessary to activate anthocyanin biosynthesis-responsive genes (Supplementary Fig. S3).

The *SlRUP* gene functions as a negative regulator of UV-B light signaling responses by promoting the reversion of the active *SlUVR8* monomer into its inactive homodimer form, thereby modulating photomorphogenic responses (Zhang et al., 2021). Given that the expression levels of SlUVR8 identified in this study suggest its role as a key regulator of the anthocyanin biosynthesis pathway, *SlRUP* may act as a potential negative regulator of this pathway due to its repressive effect on SlUVR8. In our study, the expression levels of *SlRUP* were responsive to light (Fig. 5; Supplementary Table S8), consistent with the observations in the Ailsa Craig genotype developed under light conditions (Zhang et al., 2021). Apparently, in the MT-*Aft/atv/hp2* genotype, the presence of *SlRUP* was not enough to influence the lack of anthocyanin accumulation in fruit tissues. However, future investigations into the role of this gene in regulating *SlUVR8* conformation, particularly anthocyanin-enriched genotypes such as MT-*Aft/atv/hp2* may contribute to enhancing the accumulation of this compound in fruit tissues. Furthermore, it is important to explore the stability of *SlRUP* in these genotypes, considering they carry alleles from wild species, which may have led to structural alterations in *SlRUP* that affect its ability to redimerize *SlUVR8*.

4.4 Transcriptional patterns of anthocyanin-positive regulatory genes may explain the lack of anthocyanin synthesis in the mesocarp

We demonstrated that anthocyanin accumulation in MT-*Aft*/*atv*/*hp2* fruits is light-dependent. Tomato lines with the dominant *Aft* (*Anthocyanin fruit*) allele display a spotted-purple phenotype linked to a genomic region that contains four in-tandem R2R3 MYB genes (Sapir et al., 2008; Cao et al., 2017): *SlAN2* (*Solyc10g086250*), *SlANT1* (*Solyc10g086260*), *SlANT1-like* (*Solyc10g086270*), and *SlAN2-like* (*Solyc10g086290*), which corresponds to the *AFT* gene. Fig. 5A shows that *SlANT1*, *SlANT1-like*, and *SlAN2* displayed no or very low expression levels in both cyanic and acyanic fruit tissues. Similar studies on tomato genotypes

with the *Aft* locus found insignificant expression levels for these genes (Qiu et al., 2019; Colanero et al., 2020b; Sun et al., 2020), showing that they are not involved in regulating anthocyanin biosynthesis in *Aft*-bearing purple tomato fruits.

In contrast, *SlAN2-like* was highly expressed in all tissues and conditions analyzed. Its expression was only slightly lower in the non-pigmented epicarp at 0d of exposure to light compared to the purple epicarp of fruits developed under normal light conditions (Fig. 5B). The same pattern was reported for the 'Indigo Rose' cultivar and a mutant with a reduced anthocyanin pigmentation (Qiu et al., 2019).

The MYB transcription factor SlAN2-like interacts with the constitutive factors bHLH1 (SlJAF13) and WDR (SlAN11) to form the first MBW complex, which induces *bHLH2* (*SlAN1*) expression. Subsequently, bHLH2 (SlAN1) replaces bHLH1 (SlJAF13) to configure the second MBW complex. This second complex, in turn, activates the expression of *SlAN1* ("reinforcement mechanism") and the LBGs (Colanero et al., 2020b). Even though *SlJAF13* and *SlAN11* expression levels fluctuated across samples, they were constitutively detected in pigmented and non-pigmented tissues (Fig. 5A), which was also observed in other studies (Gao et al., 2018; Gonzali et al., 2025). Transcriptional analysis in different purple tomato lines showed that *SlAN1* expression is correlated with the level of anthocyanin pigmentation (Butelli et al., 2008; Bassolino et al., 2013; Qiu et al., 2016, 2019; Gonzali et al., 2025), which was confirmed in our study (Fig. 5A). Our findings indicate that *SlAN1* may be the limiting factor governing the development of anthocyanin pigmentation of tomato fruit tissues, with its expression being light-dependent.

Although *SlAN2-like* was the most highly expressed anthocyanin-related MYB factor across all tissues and conditions analyzed, its expression levels remained relatively stable in both cyanic and non-cyanic tissues of tomato fruits at 30-35 days after anthesis (Fig. 5A). These findings led us to speculate about a possible negative regulation of the SlAN2-like protein by COP1 in tomato fruit tissues under dark conditions, similar to what is observed in apples and eggplants (Li et al., 2012, 2024).

Transcription factors from the WRKY family are essential regulators of anthocyanin biosynthesis in an HY5-independent manner (Qiu et al., 2019). WRKY physically interacts with WD to form a transcriptional complex independent of the MBW complex, leading to the transcriptional activation of membrane transporter and vacuolar acidification genes (Lloyd et al., 2017). In our study, the *SlWRKY* transcriptional profile matches the anthocyanin

accumulation pattern in MT-*Aft/atv/hp2* fruit tissues (Fig. 5A), which was also observed by Gonzali *et al.,* 2025. Thus, our model suggests that *SlWRKY* expression is regulated by light and that it plays an essential cyanogenic role in tomato fruits by activating the transcription of anthocyanin structural genes.

4.5 COP1 may act as a negative regulator of SlAN2-like activity in tomato fruit tissues developed in the dark

The interaction between COP1 and MYB proteins in regulating anthocyanin accumulation has been observed in both apple and eggplants. In apple, MdCOP1 interacts with MdMYB1 to regulate light-induced anthocyanin biosynthesis (Li et al., 2012), while in eggplants, SmCOP1 interacts with SmMYB5, promoting its degradation via the 26S proteasome pathway (Li et al., 2024). The gene expression patterns of *SlCRY3*, *SlUVR8*, *COP1 homolog*, *COP1-like isoform X1*, and *SlAN2-like* observed in this study (Fig. 4B and 5) suggest that similar interactions may also occur in tomato fruits, directly influencing anthocyanin accumulation.

Both *COP1* genes, the *COP1 homolog* and the *COP1-like isoform X1*, showed substantial expression levels in the epicarp and mesocarp tissues of the MT-*Aft/atv/hp2* genotype under the conditions analyzed in this study. Additionally, Menconi et al., (2025) reported expression of both genes in the MT-*Aft/atv/hp2* and MT-*Aft/atv* genotypes, along with evidence of protein activity in protoplasts. These findings indicate that both genes have the potential to function as COP1 in tomato plants. Nevertheless, further function analyses are required to determine which of the two plays the dominant role in protein degradation under dark conditions in tomato fruit tissues.

Despite the high expression of *SlAN2-like* under dark conditions (acyanic tissues; Fig. 5A), its activity might be post-translationally suppressed by COP1. Under light conditions, however, the transcription of *SlCRY3* and *SlUVR8* is upregulated (Fig. 4B), indicating that these photoreceptors may become the primary targets of COP1, allowing SlAN2-like to escape repression. In summary, under dark conditions, COP1 likely functions as a negative regulator of the SlAN2-like protein, preventing the assembly of the first MBW complex and, consequently, inhibiting *SlAN1* expression. Conversely, light triggers the expression of photoreceptor genes, redirecting COP1 activity toward these newly available targets. This

redirection allows the formation of the first MBW complex (SlAN2-like/SlJAF13/SlAN11), ultimately activating *SlAN1* expression (Fig. 7).

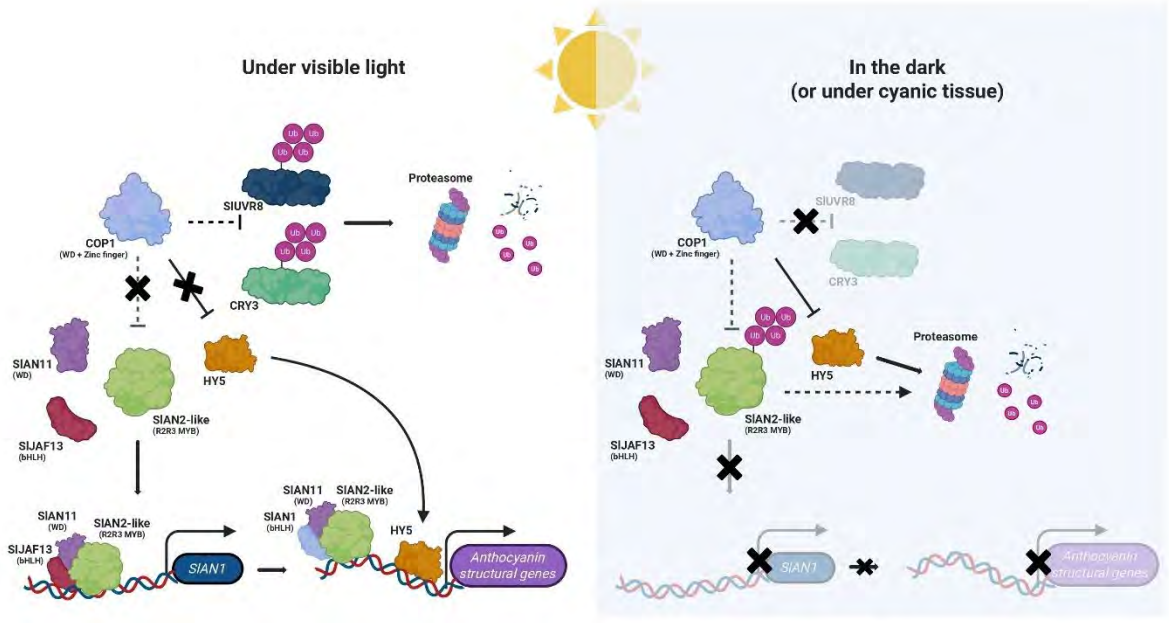

Fig. 7. Possible transcriptional model for anthocyanin biosynthesis regulation under light and dark conditions.

Under visible light, instead of ubiquitinating the SlAN2-like (*Solyc10g086290*), the COP1 (*Solyc12g005950*) ubiquitinates the photoreceptors SlUVR8 (*Solyc05g018630*) and CRY3 (*Solyc08g074270*), leading to their degradation by the proteasome. In this condition, the SlAN2-like forms the first MBW complex together with the SlJAF13 (*Solyc08g081140*) and SlAN11 (*Solyc03g097340*) to activate the *SlAN1* (*Solyc09g065100*) gene expression. After that, the SlAN1 replaces the SlJAF13 to form the second MBW complex to activate the anthocyanin structural genes. In dark conditions, COP1 will ubiquitinate the SlAN2-like, inducing its degradation by the proteasome, inhibiting the formation of the first MBW complex by activating the *SlAN1* expression and, consequently, the anthocyanin biosynthesis.

Although the DET1 mutation, present in *hp2* genotypes, compromises the functional integrity of the COP1-mediated protein complex (Cañibano et al., 2021), the possibility that this system retains some residual activity in some genes cannot be ruled out. It is important to note that this knowledge is derived from studies conducted in *Arabidopsis thaliana*, and its direct extrapolation to *Solanum lycopersicum* should be approached with caution, given the differences in the composition and regulation of COP1 and DET1 factors between species. Furthermore, our GO term analysis revealed an enrichment of protein ubiquitination (GO:0016567) at day 0 in the acyanic epicarp, compared to fruits exposed to normal light, as

well as in the epicarp compared to the mesocarp of control samples (Supplementary Fig. S5). Therefore, based on the transcriptional profile of the genes analyzed here and the anthocyanin pigmentation pattern, our hypothesis is promising and warrants further investigation, including direct experimental validation at the protein level.

4.6 Structural genes and MYB repressor expression in the MT-*Aft/atv/hp2* genotype

Genes that encode enzymes of the anthocyanin pathway are divided into "*early biosynthetic genes*" (EBGs) and "*late biosynthetic genes*" (LBGs; Quattrocchio et al., 2006). EBGs are related to synthesizing flavonoid precursors and final products e.g., chalcones, dihydroflavonols, and flavonols. In contrast, LBGs are more specific for anthocyanins (Fig. 6A). In our study, the expression pattern of anthocyanin structural and regulatory genes correlated with anthocyanin pigmentation in the tissues of tomato fruits (Fig. 6B), the same expression pattern observed by Gonzali et al., 2025.

In addition to activating *SlAN1* and LBGs, the second MBW complex also induces the expression of MYB repressors (Albert et al., 2014; Colanero et al., 2020b). The R3-MYB protein can directly bind to the bHLH factors, inhibiting the formation of the MBW complex activator of anthocyanin-related genes (Colanero et al., 2018; Sun et al., 2020).

SlMYB-ATV (*Solyc07g052490*) inhibits anthocyanin biosynthesis in tomato fruits (Cao et al., 2017; Colanero et al., 2018). Its transcriptional activation is triggered by the MBW ternary complex (SlAN2-like/SlAN1/SlAN11), which also promotes *SlAN1* expression (Colanero et al., 2018). In our study, the expression pattern of *SlMYB-ATV* mirrors that of *SlAN1* (Fig. 5A), indicating that both genes are activated by the same complex. The functional, dominant *SlMYB-ATV* allele exists in *S. lycopersicum*, while the recessive *atv* allele is found in some wild species, such as *S. cheesmaniae* (Cao et al., 2017). The *atv* allele produces a truncated, non-functional protein due to a 4-bp insertion in the second exon, causing a frameshift and a premature stop codon. This results in a truncated SlMYB-ATV protein lacking the R3-domain, which cannot bind bHLH factors and thus cannot disrupt the MBW complex (Cao et al., 2017; Colanero et al., 2018; Sun et al., 2020), leading to increased anthocyanin levels in tomato tissues. Therefore, the nonfunctional *SlMYB-ATV* allele promotes anthocyanin pigmentation in tomato fruit tissues.

4.7 Additional traits observed in MT-*Aft/atv/hp2* plants

The yield of MT-*Aft/atv/hp2* plants was reported to be comparable to that of cv. MT. MT-*Aft/atv/hp2* fruits showed significantly higher levels of ascorbic acid, lycopene, and β-carotene than cv. MT (Sestari et al., 2014). Anthocyanins have also been associated with increased resistance to biotic stresses in mango (Sivankalyani et al., 2016). We observed that MT-*Aft/atv/hp2* plants appeared more resistant to thrips (*Thysanoptera*) than MT plants in greenhouse conditions. Even though we have not addressed this question in our current research, it warrants further investigation.

5. Conclusions

This study brings novel information on anthocyanin metabolism in the mesocarp cells of purple tomato fruits mediated by light. Short wavelengths, such as blue and UV-B light, represent crucial signals for anthocyanin biosynthesis activation in cyanic tomato fruits. In the epicarp of MT-*Aft/atv/hp2* fruits, these wavelengths are detected early during development and trigger signal transduction pathways, thereby inducing the expression of the anthocyanin regulatory and structural genes, resulting in anthocyanin accumulation. This pigmentation establishes a filter that blocks the short wavelengths from reaching the deep tissues of the fruit, consequently suppressing the expression of the *SlAN1*, a likely limiting gene for anthocyanin accumulation. Exposing acyanic fruits, which develop in the dark, to light for five days promotes anthocyanin accumulation in the epicarp and the mesocarp tissues. Therefore, our results strengthen a working hypothesis to elucidate the anthocyanin recalcitrance observed in parenchymatic cells of inner fruit tissues. To overcome this resistance, we propose a reliable approach or genetic pathway preventing early epidermis pigmentation.

Author contributions

G.L.R., A.L.S., L.E.P.P., A.C.-Jr., and V.A.B. conceived and planned the study. G.L.R., A.L.S., and V.A.B. designed the experiments. G.L.R., S.C.-S., and A.L.S. performed the experiments. G.L.R., C.F.V., L.W.P.A., and V.A.B. analyzed the data. G.L.R., C.F.V., and L.W.P.A. wrote the manuscript. V.A.B., A.Z., L.E.P.P., and A.C.-Jr. revised the manuscript. V.A.B. supervised all steps of the study and final manuscript. All authors read and approved the manuscript.

Conflict of Interest

No conflict of interest declared.

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Data availability statement

The RNA-seq data underlying this article are available in the Gene Expression Omnibus (GEO) Database (GSE235565).

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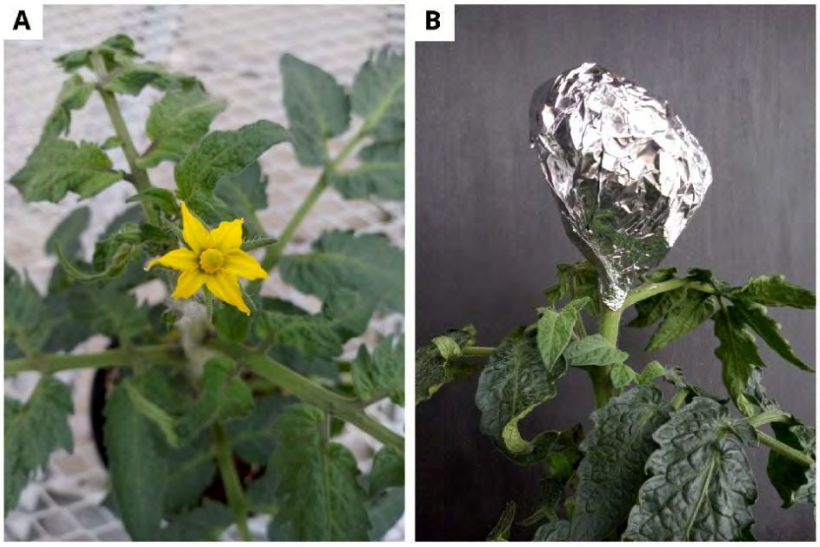
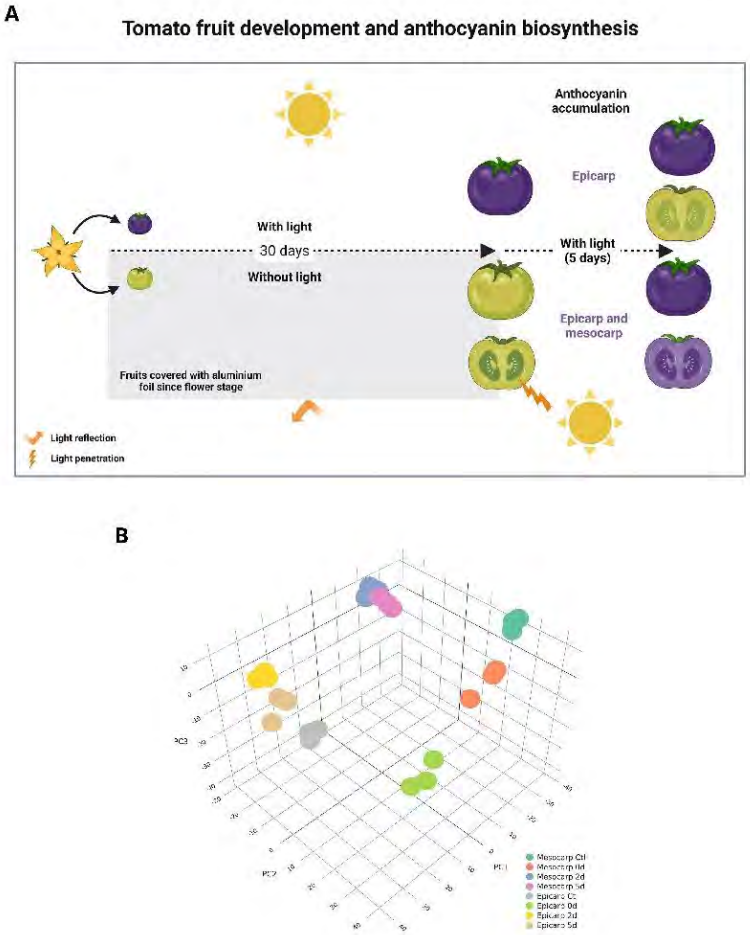
Supplementary Material

Supplementary Figures

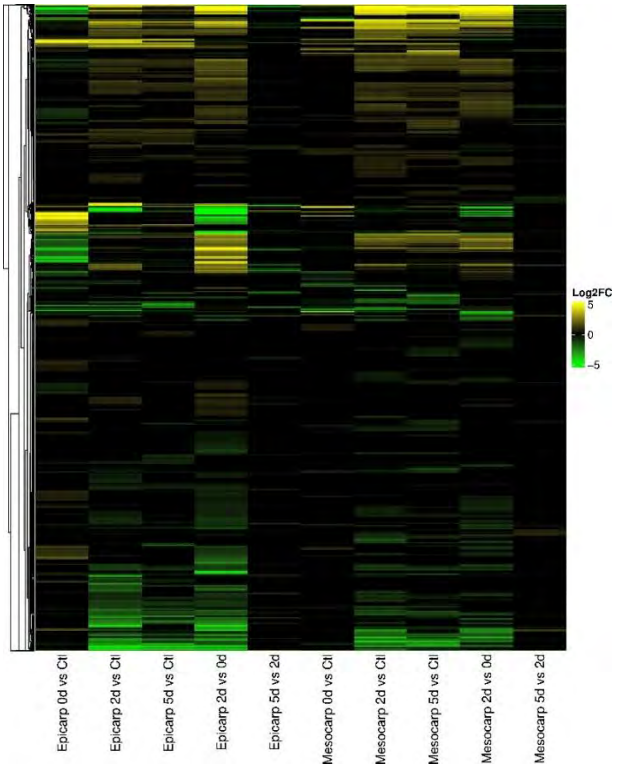


Supplementary Fig. S1 Restriction of light incidence during fruit development. Individual flowers were covered with aluminum foil at anthesis **(A)** for 30 days **(B)**.

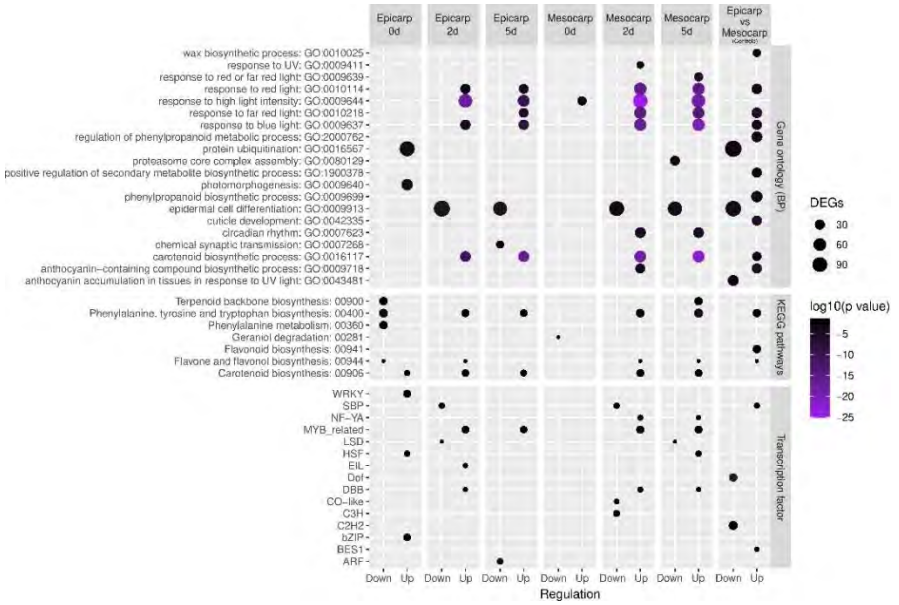
Supplementary Fig. S2 Characterization of the progressive development of anthocyanin pigmentation in the epicarp of MT-*Aft/atv/hp2* under different light conditions for 30 days after anthesis. **(A)** Thirty-day fruit developed in the dark immediately after cover removal. **(B-F)** Covered fruit exposed to normal light conditions for 1**(B)**, 2**(C)**, 3**(D)**, 4**(E)**, and 5**(F)** days after cover removal. **(G)** Fruit developed in normal light conditions (not covered).



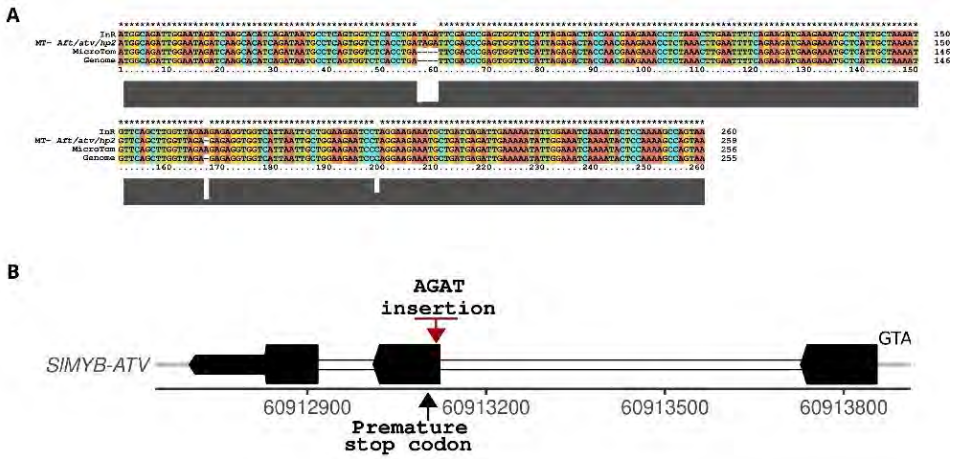
Supplementary Fig. S3 (A) Summary of the experimental design used to manipulate light and its effect on fruit anthocyanin accumulation. At the anthesis, tomato flowers were submitted to light and dark conditions for 30 days. In light conditions, the tomato fruit showed cyanic epicarp and acyanic mesocarp. In the dark, the fruits were entirely acyanic. After the cover removal, the acyanic fruits were exposed to light for 5 days, leading to anthocyanin accumulation in the epicarp and mesocarp of these fruits. **(B)** Principal component analysis based on expression values.



Supplementary Fig. S4 Heatmap of DEGs. Each column compares two conditions, whereas each line represents a gene. See Table S3 for details. d, days; Ctl, control (fruit grown under light conditions); Log2FC, logarithmic base 2 of fold change.



Supplementary Fig. S5 Analyses of Gene Ontology (GO) terms, KEGG pathways, and transcription factors enriched in the differentially expressed gene (DEG) set within each tissue at a specific light exposure time compared to the control condition (fruit developed under normal light exposure). The last comparison shows DEG regulation between the epicarp and mesocarp in the control fruit. Ctl, control; d, days of exposure to light after cover removal.



Supplementary Fig. S6 (A) Sequence alignment of *SlMYB-ATV* (*Solyc07g052490*) transcripts from cv. 'Indigo Rose' (InR), MT-*Aft/atv/hp2*, Micro-Tom, and Heinz (genome) tomato genotypes. **(B)** Schematic representation of the *SlMYB-ATV* (*Solyc07g052490*) gene mutation in the MT-*Aft/atv/hp2*.

Supplementary Tables

Table S1. Summary of cleaning, mapping, and counting of reads on the tomato reference genome.

	Trimmomatic (trimming)			STAR (mapping)			featureCounts (counting)	
Library	Input	Output (#)	Output (%)	Input	Output (#	Output (%)	Count (#)	Count (%)
Epicarp 0d - 1	24625955	23817678	96,72	2,4E+07	2,3E+07	94,82	20940821	92,73
Epicarp 0d - 2	23224760	22366775	96,31	2,2E+07	2,1E+07	94,23	19488346	92,46
Epicarp 0d - 3	22443390	21626959	96,36	2,2E+07	2E+07	92,08	18412051	92,46
Epicarp 2d - 1	25618472	25020119	97,66	2,5E+07	2,4E+07	94,41	21871092	92,59
Epicarp 2d - 2	25093982	24508653	97,67	2,5E+07	2,3E+07	94,02	21314132	92,50
Epicarp 2d - 3	24078770	23570852	97,89	2,4E+07	2,2E+07	94,21	20568018	92,62
Epicarp 5d - 1	24539050	23583511	96,11	2,4E+07	2,2E+07	92,34	20130065	92,44
Epicarp 5d - 2	24495128	23863556	97,42	2,4E+07	2,2E+07	93,71	20718849	92,65
Epicarp 5d - 3	25070292	24231332	96,65	2,4E+07	2,3E+07	94,22	21128384	92,54
Epicarp Ctl - 1	22232531	21633800	97,31	2,2E+07	2E+07	92,69	18618384	92,85
Epicarp Ctl - 2	25463534	24847731	97,58	2,5E+07	2,3E+07	94,13	21628368	92,47
Epicarp Ctl - 3	25894885	25281492	97,63	2,5E+07	2,4E+07	93,37	21853831	92,58
Mesocarp 0d - 1	22603705	22011011	97,38	2,2E+07	2,1E+07	95,01	19526720	93,37
Mesocarp 0d - 2	26846397	26279578	97,89	2,6E+07	2,5E+07	94,66	23158324	93,10
Mesocarp 0d - 3	27071532	26108777	96,44	2,6E+07	2,5E+07	94,01	22756149	92,71
Mesocarp 2d - 1	26124014	25178555	96,38	2,5E+07	2,4E+07	94,00	21904682	92,55
Mesocarp 2d - 2	23361163	22812397	97,65	2,3E+07	2,2E+07	94,49	20019109	92,87
Mesocarp 2d - 3	21756876	21013616	96,58	2,1E+07	2E+07	94,51	18402751	92,66
Mesocarp 5d - 1	25475415	24748880	97,15	2,5E+07	2,3E+07	94,22	21575300	92,53
Mesocarp 5d - 2	22300613	21740871	97,49	2,2E+07	2E+07	93,95	18925789	92,66
Mesocarp 5d - 3	27339099	26663725	97,53	2,7E+07	2,5E+07	93,93	23197867	92,62
Mesocarp Ctl - 1	22854540	22214436	97,20	2,2E+07	2,1E+07	93,97	19397406	92,92
Mesocarp Ctl - 2	22138429	21324427	96,32	2,1E+07	2E+07	93,33	18465849	92,79
Mesocarp Ctl - 3	24969585	24375453	97,62	2,4E+07	2,3E+07	94,25	21355008	92,95
Average	24400921	23701007	97,12	2,4E+07	2,2E+07	93,94	20639887	92,69
Total	585622117	568824184						

Table S2. Primers used in the RT-qPCR analyses.

Gene name	Primer Sequence (5'-3')
SlAN2-like (*MYB114*)	Fw: GACCACGACCAAGACCAG
	Rv: TTACTGCGATTTCTTTGTTGTG
Solanum U6 (*housekeeping*)	Fw: GGACATCCGATAAAATTGG
	Rv: GATTTGTGCGTGTCATCCT
5.8S (*housekeeping*)	Fw: GGGCGAGTCCAAAATCCAAT
	Rv: GGTGTTTTCACGTCTTACCGT
COP1 (*Solyc05g014130*)	Fw: GATGGAGCAACGGAAATGG
	Rv: GGAATCGGAGACAATTACAGC
SlCOP1 (*Solyc12g005950*)	Fw: CTATCCCAATTTCCTACTTGAC
	Rv: CATCAACAATAGAGCGTCCAG

Table S3. Differentially expressed genes by log2FC interval.

	Control comparison							Day comparison			
	Epicarp (days)			Mesocarp (days)			Epicarp vs	Epicarp (days)		Mesocarp (days)	
Interval of log2FC	0	2	5	0	2	5	Mesocarp	2 vs 0	5 vs 2	2 vs 0	5 vs 2
Up	2288	2813	1990	996	3024	2774	2783	3683	512	2798	519
>6	123	111	62	70	136	128	252	170	17	106	6
4~6	179	126	58	73	169	146	229	209	9	127	11
2~4	303	334	201	127	455	406	490	722	42	451	65
1~2	373	737	490	158	1009	898	647	1066	122	933	111
0~1	1310	1505	1179	568	1255	1196	1165	1516	322	1181	326
0~-1	1000	1283	911	358	1398	1255	1316	1901	424	1694	384
-1~-2	674	869	556	223	776	543	558	1038	192	610	144
-2~-4	384	628	341	99	394	287	400	757	149	329	43
-4~-6	120	179	73	25	76	59	250	285	52	122	16
<-6	93	87	29	21	37	19	511	163	18	63	5
Down	2271	3046	1910	726	2681	2163	3035	4144	835	2818	592
Total	4559	5859	3900	1722	5705	4937	5818	7827	1347	5616	1111

Table S7. Expression of the photoreceptor genes in the epicarp and mesocarp of tomato fruits (MT-Aft/atv/hp2).

		Average - log2(TPM+1)								Log2(FC)										
		Epicarp				Mesocarp				Epicarp vs Ctl			Mesocarp vs Ctl			Epicarp (Ctl) vs	Epicarp vs days		Mesocarp vs days	
Gene	Symbol	0	2	5	Ctl	0	2	5	Ctl	0	2	5	0	2	5	Mesocarp (Ctl)	2 vs 0	5 vs 2	2 vs 0	5 vs 2
Solyc10g044670	SlPHYA	5,89	6,3	5,96	5,7	5,51	6,07	5,8	5,49	0	0,64	0	0	0,88	0	0	0,53	0	0,7	0
Solyc01g059870	SlPHYB1	4,38	3,6	3,46	3,8	4,38	4,4	4	4,78	0,55	0	0	0	0	-0,6	-0,91	-0,66	0	0	-0,55
Solyc05g053410	SlPHYB2	5,12	3,4	3,77	4,6	4,9	3,86	4,2	6,29	0	-1,3	-0,9	-1,27	-2,2	-1,9	-1,58	-1,74	0	-0,95	0
Solyc04g074180	SlCRY1a	4,92	5	5,05	5	5,33	5,37	5,2	5,16	0	0	0	0	0,5	0	0	0	0	0	0
Solyc12g057040	SlCRY1b	6,03	7,4	7,41	6,6	6,4	7,31	7,1	6,59	0	0,86	0,8	0	1,02	0,79	0	1,45	0	1,09	0
Solyc09g090100	SlCRY2	6,24	6,5	6,58	5,9	6,32	6,77	6,7	6,23	0	0,66	0,76	0	0,81	0,69	0	0	0	0,55	0
Solyc08g074270	CRY3	2,14	4,6	3,94	3,8	1,92	4,49	3,2	2,69	-1,9	0,97	0	0	2,12	0	1,19	2,91	0	3,01	-1,39
Solyc05g018630	SlUVR8	6,63	7,2	7,22	6,9	6,17	6,67	6,8	6,27	0	0	0	0	0,64	0,72	0,69	0,63	0	0,64	0

Table S8. Expression [log (TPM+1)] and differential expression (log2FC) of the anthocyanin biosynthetic regulatory genes in the epicarp and mesocarp of tomato fruits (MT-Aft/atv/hp2).

		Average - Log2(TPM+1)								Log2(FC)										
		Epicarp				Mesocarp				Epicarp vs Ctl			Mesocarp vs Ctl			Epicarp (Ctl) vs	Epicarp		Mesocarp	
Gene	Symbol	0	2	5	Ctl	0	2	5	Ctl	0	2	5	0	2	5	Mesocarp (Ctl)	2 vs 0	5 vs 2	2 vs 0	5 vs 2
Solyc01g095640	SlTRY	0	0,79	1,65	0,4	0,07	0,25	0,31	0	0	0	0	0	0	0	0	0	0	0	0
Solyc09g065100	SlAN1|AH	0,46	7,98	7,27	6,29	0,21	7,05	6,72	0,21	-7,72	1,75	1,05	0	10,02	9,66	9,1	9,47	0	9,89	0
Solyc10g086250	SlAN2	0,97	0,63	0,68	1,54	0,04	0,12	0,04	0,78	0	0	0	0	0	-5,07	0	0	0	0	0
Solyc10g086260	SlANT1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Solyc10g086270	SlANT1-like	0,4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Solyc10g086290	SlAN2-like	7,96	10,89	11,09	10,63	6,29	10,5	10,29	8,23	-2,03	0	0	0	2,55	2,28	2,53	2,32	0	3,69	0
Solyc03g097340	SlAN11	6,35	9,57	8,4	7,63	6,18	7,94	7,98	6,43	-1,36	2	0,84	0	1,81	1,82	1,35	3,36	-0,56	1,95	0
Solyc08g081140	SlJAF13	4,47	4,8	4,45	4,21	4,51	5,5	4,53	4,33	0	0,66	0	0	1,5	0,46	0	0,48	0	1,18	-1,05
Solyc01g056340	SlDET1	2,21	1,97	2,23	1,95	2,03	2,05	1,92	2,07	0	0	0	0	0	0	0	0	0	0	0
Solyc07g052490	SlMYB-ATV	0,63	8,78	7,67	5,24	0	8,08	7,08	0,62	-5,97	3,64	2,54	-4,63	9,35	8,25	6,24	9,61	0,61	13,98	-1,1
Solyc12g005795	SlMYB-ATV-like (Solyc12g005800)	0,18	4,83	4,97	4,29	0	0,98	3,46	0	-6,91	0	0	0	6,39	9,73	10,41	7,55	0	6,46	3,34
Solyc05g008250	SlMYBL2|SlMYB76	0,04	5,74	6,98	6,21	0,04	2,89	5,57	0,46	-11,19	0	0	0	4,4	7,24	7,59	10,77	0	8,15	2,84
Solyc06g065100	SlMYB3	5,33	5,74	5,8	5,59	3,85	4,42	4,29	4,34	0	0	0	0	0	0	1,56	0	0	0	0
Solyc01g111500	SlMYB7	2,4	4,34	3,93	3,13	2,7	4,21	3,57	2,13	0	1,23	0	0	2,61	1,9	1,46	2,32	0	1,8	0
Solyc10g055410	SlMYB32	5,55	8,11	7,24	6,97	4,22	6,5	6,01	4,52	0	0	0	0	2,24	1,67	2,58	2,22	0	2,16	0
Solyc03g120620	HBZIP (GLABRA)	3,02	7,62	6,38	6,56	3,23	5,84	5,47	4,22	-3,53	1,12	0	0	1,89	1,51	2,47	4,65	-0,58	2,89	0
Solyc10g084380	WRKY (TTG2)	1,21	8,29	7,09	6,15	0,29	5,1	5,18	0,65	-5,33	0	0	0	6,07	6,03	6,9	7,54	0	7,19	0
Solyc12g005950	SlCOP1	4,52	4,44	4,48	4,16	4,51	3,96	4,19	4,5	0	0	0	0	0	0	0	0	0	-0,43	0
Solyc05g014130	COP1	3,88	3,11	3,45	3,27	4,03	3,66	3,49	4,34	0	0	0	0	0	-0,72	-1,08	-0,71	0	0	0
Solyc08g061130	SlHY5	5,7	3,74	3,05	4,21	4,67	3,98	2,17	2,78	2,03	0	0	2,54	1,58	0	1,7	-2,48	0	0	-2,14

Table S9. Expression [log (TPM+1)] and differential expression (log2FC) of the anthocyanin biosynthetic structural genes in the epicarp and mesocarp of tomato fruits (MT-*Aft/atv/hp2*).

		Average - Log2(TPM+1)								Log2(FC)										
		Epicarp				Mesocarp				Epicarp vs Ctl			Mesocarp vs Ctl			Epicarp (Ctl)	Epicarp		Mesocarp	
Gene	Symbol	0	2	5	Ctl	0	2	5	Ctl	0	2	5	0	2	5	vs Mesocarp (Ctl)	2 vs 0	5 vs 2	2 vs 0	5 vs 2
Solyc09g007900	PAL5	6.50	9.83	10.84	9.66	3.73	9.57	10.46	5.23	-3.04	0	1.24	-1.43	4.63	5.52	4.58	3.27	0	06.06	0
Solyc05g047530	C4H	0.75	3.60	2.79	3.74	0.48	0.09	0.99	####	-4.17	0	0	0	-3.81	0	3.66	3.99	0	0	4.13
Solyc06g068650	4CL	1.91	7.00	6.99	5.42	2.56	5.31	6.39	3.99	-3.27	0	0	0	0	2.71	0	4.89	0	2.51	0
Solyc09g091510	CHS1	3.25	12.31	11.21	10.71	0.84	11.29	10.82	####	-7.43	1.64	0	0	11.37	10.89	10.63	09.06	0	11.77	0
Solyc05g053550	CHS2	8.40	13.21	####	12.63	04.06	####	11.86	####	-2.96	0	0	0	9.43	9.26	9.87	3.59	0	6.36	0
Solyc05g010320	CHI	2.87	8.79	7.67	6.19	3.61	8.59	9.14	####	-3.03	2.68	1.63	0	4.96	5.43	2.35	5.72	0	5.16	0
Solyc05g052240	CHI-LIKE	4.46	10.93	10.40	9.20	1.84	9.42	9.65	####	-4.59	1.78	1.28	0	####	8.20	7.58	6.37	0	8.28	0
Solyc02g083860	F3H	8.99	12.89	12.17	11.38	4.30	12.50	11.89	6.20	-2.45	1.56	0	-1.75	6.34	5.75	05.08	4.00	0	08.09	0
Solyc03g115220	F3'H	3.55	7.97	7.46	5.73	1.65	5.27	####	2.00	-2.38	2.26	1.71	0	3.94	####	4.29	4.63	0	4.27	-1.91
Solyc11g013110	FLS	####	9.58	9.38	9.66	4.30	7.41	5.48	6.48	0	0	0	0	0	0	3.31	0	0	2.58	-1.88
Solyc11g066580	F3'5'H	0.57	####	10.60	9.98	0.37	9.67	9.86	0.75	-11.06	1.18	0	0	10.47	10.71	10.63	12.23	0	11.59	0
Solyc02g085020	DFR	1.24	12.39	11.65	10.72	0.79	11.81	11.44	1.85	-10.35	1.72	####	-1.68	10.70	10.33	9.47	12.07	0	12.39	0
Solyc08g080040	ANS	2.84	12.73	11.87	10.74	2.77	12.59	####	3.44	-8.05	####	1.25	0	9.56	####	7.57	10.08	0	10.20	0
Solyc10g083440	3GT	9.47	12.28	11.66	11.14	4.51	11.87	####	5.37	-1.75	1.19	0	0	6.72	5.90	5.85	2.94	0	7.14	0
Solyc09g059170	RT	4.72	13.65	12.62	12.00	2.85	12.34	####	4.95	-7.23	1.70	0	-2.13	7.50	7.26	07.01	8.93	0	9.63	0
Solyc12g088170	AAC	1.40	####	11.20	10.26	0.62	11.91	11.15	1.28	-9.44	1.85	0	0	11.56	10.79	9.75	11.28	0	12.89	0
Solyc09g092500	5GT	7.71	5.99	6.27	5.29	7.45	8.32	7.91	6.47	2.91	0	0	0	2.23	0	0	-2.09	0	0	0
Solyc02g081340	GST	1.19	12.45	11.92	11.25	01.06	12.18	11.58	1.74	-10.97	1.25	0	0	####	10.51	10.02	12.22	0	12.15	0
Solyc03g025190	PAT	####	11.60	####	####	0.54	10.95	10.71	0.98	-9.92	1.58	####	0	11.20	10.97	10.17	11.51	0	12.15	0
Solyc12g010980	HCT	0.51	1.89	0.32	0.13	0.41	0.48	1.44	0.86	0	####	0	0	0	0	0	2.88	-3.61	0	0
Solyc01g096670	C3'H	2.36	6.97	6.12	5.65	1.79	6.96	6.49	3.34	-3.59	1.41	0	-1.76	3.90	3.37	2.41	4.99	0	5.66	0
Solyc02g093270	CCoAOMT	5.15	6.77	6.33	6.29	3.89	4.91	5.89	5.86	0	0	0	0	0	0	0	1.62	0	0	0

Artigo 2 - Redigido conforme norma do periódico científico - Versão preliminar	
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ARTICLE 2 – Beyond HY5: COP1 posttranslational control of anthocyanin biosynthesis proteins in horticultural crops**

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Abstract

Anthocyanins are widespread specialized metabolites that provide pigmentation and antioxidant capacity, contributing to pollinator and seed-disperser attraction and to plant resistance to diverse environmental stresses. In human diets, anthocyanins are valued for their antioxidant and health-promoting properties. The biosynthetic pathway of anthocyanins is relatively conserved across plant species and is controlled by structural genes that encode the enzymes of the pathway along with regulatory genes, particularly transcription factors. This network integrates developmental and environmental signals, with light serving as a dominant cue: anthocyanins typically accumulate in light-exposed tissues and are repressed in darkness. A key node in this light-dependent switch is CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1), an E3 ubiquitin ligase that, in the dark, promotes polyubiquitination and proteasome-mediated turnover of positive regulators of anthocyanin production. Although ELONGATED HYPOCOTYL 5 (HY5) is a canonical COP1 target and major activator of anthocyanin biosynthesis, COP1 control of this pathway extends well beyond HY5. Evidence from Arabidopsis and multiple horticultural crops, including apple, pear, eggplant, and tomato,

indicates that COP1 also regulates anthocyanin accumulation through interactions with additional transcription factors and regulatory modules. Here, we synthesize recent advances in COP1-centered regulation of anthocyanin biosynthesis, with an emphasis on post-translational mechanisms and COP1 targets beyond HY5. We also discuss emerging opportunities to leverage this regulatory axis for nutritional improvement in horticultural species.

Keyworks: bioactive compound; light; post-translational regulation; ubiquitination

1. Introduction

Anthocyanins are specialized flavonoid pigments derived from the phenylpropanoid pathway, widely distributed across the plant kingdom. Initially recognized for their ecological roles in attracting pollinators and seed dispersers, anthocyanins are now understood to contribute significantly to plant stress resistance and photoprotection [1]. Beyond their physiological functions in plants, dietary anthocyanins from fruits and vegetables have garnered attention for their potent antioxidant, anti-inflammatory, and metabolic regulatory properties, with implications for cardiovascular health, diabetes management, and cancer prevention [2–4]. Nevertheless, questions regarding their bioavailability and optimal intake remain unresolved [5,6].

Anthocyanin biosynthesis is orchestrated through a well-characterized metabolic pathway, culminating in glycosylated anthocyanin derivatives. This pathway is tightly regulated by MYB-bHLH-WD40 (MBW) transcriptional complexes, which modulate gene expression in response to developmental cues and environmental stimuli [7]. Among these stimuli, light serves as a pivotal regulator of anthocyanin accumulation. Plants have evolved intricate signaling networks to perceive fluctuations in light quality, intensity, and duration, involving photoreceptors such as UVR8, cryptochromes, and phytochromes, and downstream transcriptional regulators including HY5, COP1, and MBW complexes [8–10].

The E3 ubiquitin ligase COP1 (CONSTITUTIVE PHOTOMORPHOGENIC 1) plays a central role in light/dark signaling by targeting transcription factors for degradation via the 26S proteasome in the dark, thereby repressing anthocyanin biosynthesis [11]. Upon light exposure, COP1 is excluded from the nucleus, relieving repression and enabling transcriptional activation of anthocyanin biosynthetic genes [12]. In the model species *Arabidopsis thaliana*, the bZIP transcription factor HY5 (ELONGATED HYPOCOTYL 5) is a well-established COP1

substrate [13]. The *cop1-4* mutant accumulates elevated anthocyanin levels, and analysis of the *cop1-4 hy5-215* double mutant reveals that anthocyanin biosynthesis can proceed via HY5-independent mechanisms, suggesting that COP1 may regulate additional components of the pathway [14].

The central role of HY5 in anthocyanin biosynthesis has been documented [9,15–17]. However, emerging evidence points to a broader regulatory landscape in which COP1 modulates multiple transcriptional nodes. This review aims to explore the multifaceted role of COP1 in anthocyanin biosynthesis, highlighting alternative regulatory routes and potential targets. A deeper understanding of these points of control may inform strategies to enhance anthocyanin accumulation in diverse crop species.

2. Anthocyanin benefits for plants and human health

Anthocyanins are water-soluble flavonoid pigments whose coloration and biological activity are governed by the flavylium cation core structure [18]. Among the six most prevalent anthocyanidins (cyanidin, delphinidin, malvidin, peonidin, petunidin, and pelargonidin), cyanidin 3-*O*-glucoside and cyanidin 3-*O*-rutinoside are particularly abundant and widely distributed in nature [19]. These compounds are responsible for the vibrant pink, red, purple, and blue hues of a variety of plant tissues, including leaves, flowers, and fruits, many of which are consumed as part of the human diet [20].

Anthocyanins are bioactive, non-essential dietary components with health-promoting properties that extend beyond their role as natural colorants. Their concentration in plant tissues is modulated by genetic background, environmental conditions, and developmental stage. Rich dietary sources include red and purple berries, grapes, apples, plums, and red cabbage, with berries, particularly blueberries and blackberries, recognized as among the most concentrated sources [21]. To date, over 1,000 distinct anthocyanin structures have been identified, underscoring their structural diversity and therapeutic potential [20].

The health benefits associated with anthocyanin consumption are well documented and include enhanced vascular function [22], improved glycemic control and diabetes prevention [23,24], anti-inflammatory activity [25,26], neuroprotection [18], and cancer prevention [2,26]. These effects are primarily attributed to their antioxidant mechanisms, which encompass free radical scavenging, activation of endogenous antioxidant enzymes, and metal ion chelation [27]. The efficacy of these mechanisms is closely linked to anthocyanin concentration and chemical structure.

As evidence for their health-promoting effects continues to grow, anthocyanins are increasingly recognized as valuable dietary components. Their widespread occurrence in fruits and vegetables makes them an accessible and safe strategy for disease prevention and health maintenance.

In addition to their relevance in human nutrition, anthocyanins play critical roles in plant physiology. They are among the most prominent specialized metabolites, contributing to oxidative stress mitigation, reactive oxygen species (ROS) scavenging, UV protection, and enhanced growth under adverse environmental conditions [28–31]. Their pigmentation also facilitates ecological interactions by attracting pollinators and seed dispersers [32]. Anthocyanins fulfill diverse ecological functions that have contributed to their evolutionary persistence. Their roles in photoprotection and plant-animal interactions underscore their adaptive significance. The dynamic regulation of anthocyanin biosynthesis in response to environmental cues provides plants with a versatile survival strategy [33].

Under abiotic stress, plants frequently upregulate anthocyanin biosynthesis as part of their adaptive response. Elevated anthocyanin levels bolster antioxidant defenses and stabilize cellular structures, improving resistance to drought, salinity, temperature extremes, and heavy metal exposure [1,34]. These compounds have also been suggested to enhance photosynthetic efficiency under high light conditions and mitigate nutrient stress, particularly under phosphorus-deficient environments [35].

Given their dual importance in promoting human health and enhancing plant resilience, elucidating the regulatory networks that control anthocyanin biosynthesis may facilitate the development of anthocyanin-enriched crops through conventional breeding or metabolic engineering

3. The general anthocyanin biosynthesis pathway

Anthocyanin biosynthesis is governed by a coordinated network of structural and regulatory genes that respond dynamically to developmental signals and environmental stimuli. The pathway initiates with the deamination of phenylalanine to cinnamic acid, catalyzed by phenylalanine ammonia-lyase (PAL), followed by hydroxylation via cinnamate 4-hydroxylase (C4H) and activation by 4-coumarate:CoA ligase (4CL), yielding 4-coumaroyl-CoA. This intermediate enters the flavonoid biosynthetic route, where chalcone synthase (CHS) condenses it with three malonyl-CoA units to produce naringenin chalcone. Chalcone isomerase (CHI) then facilitates stereospecific cyclization to form naringenin, which is hydroxylated at the C-3

position by flavanone 3-hydroxylase (F3H), generating dihydroflavonols. These steps are catalyzed by enzymes encoded by early biosynthetic genes (EBGs) (Figure 1) [7]. Subsequent reactions are mediated by late biosynthetic genes (LBGs). Dihydroflavonols undergo further hydroxylation by flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H), which determines the hydroxylation pattern of the B-ring and influences anthocyanin coloration. The final steps involve dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), and UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT), which convert dihydroflavonols into anthocyanidins and subsequently into stable glycosylated anthocyanins [7]. At the dihydroflavonol node, flux can also be diverted toward flavonol formation via flavonol synthase (FLS), competing with anthocyanin production. Additional glycosylation steps (e.g., 5-*O*-glycosyltransferases, 5-GT) further diversify and stabilize anthocyanins. Anthocyanin biosynthesis occurs in the cytosol, and then these pigments are transported to the vacuole for storage. This transport is mediated by glutathione S-transferase (GST) and/or putative anthocyanin transporter (PAT), where anthocyanins are stored [36] (Figure 1).

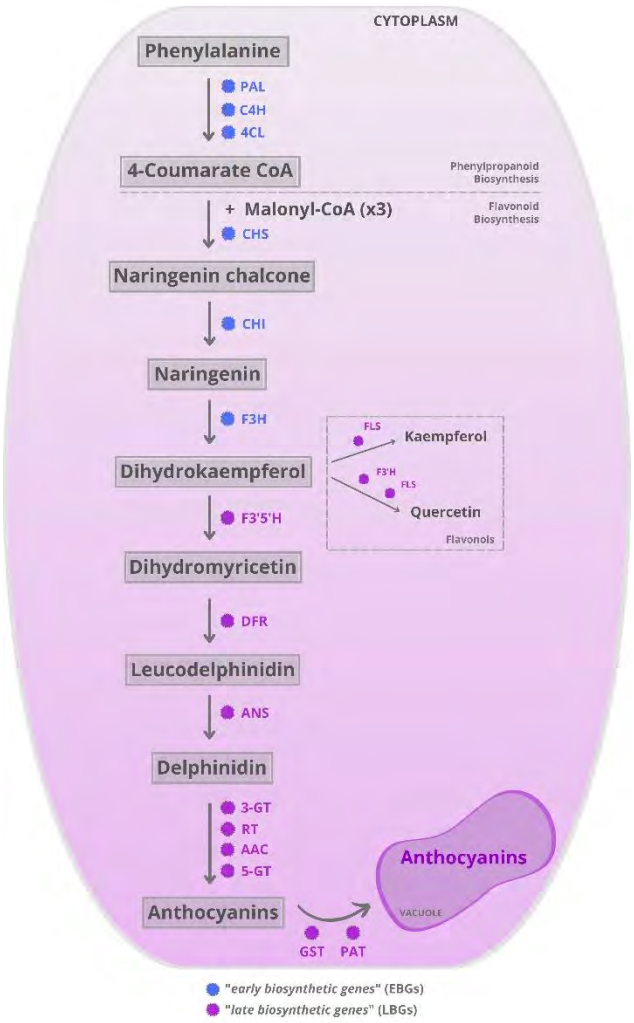

Figure 1. Anthocyanin biosynthesis pathway and vacuolar sequestration involves the coordinated enzymatic steps and transport, including early biosynthetic genes (EBGs, in blue font): phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate:CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), followed by the late biosynthetic genes (LBGs, in magenta font): flavonol synthase (FLS), flavonoid 3′-hydroxylase (F3′H), flavonoid 3′,5′-hydroxylase (F3′5′H), dihydroflavonol 4-reductase (DFR), anthocyanidin synthase (ANS), UDP-glucose:flavonoid 3-*O*-glucosyltransferase (3-GT, also known as UFGT), anthocyanin 5-*O*-glucosyltransferase (5-GT), glutathione S-transferase (GST), and a putative anthocyanin transporter (PAT).

Transcriptional regulation of anthocyanin biosynthesis is primarily mediated by the MYB–bHLH–WD40 (MBW) complex, a conserved regulatory module across plant species. This ternary complex comprises R2R3-MYB transcription factors with dual DNA-binding domains conferring gene specificity, basic helix-loop-helix (bHLH) proteins that enhance DNA binding and complex stability, and WD40 repeat proteins that function as scaffolding elements [37,38].

In *Arabidopsis*, key MYB activators include PAP1 (MYB75), PAP2 (MYB90), and MYB113, which upregulate anthocyanin biosynthetic genes. Their bHLH partners, TT8, GL3, and EGL3, possess basic DNA-binding regions and helix-loop-helix domains that mediate protein–protein interactions. The WD40 protein TTG1 stabilizes the complex and facilitates its assembly [39].

Once formed, the MBW complex binds to the promoter regions of anthocyanin-related genes by recognizing specific cis-regulatory motifs, activating transcription of both EBGs and LBGs, with particular emphasis on DFR, ANS, and UFGT. In species such as tomato (*Solanum lycopersicum*), the MBW complex may also regulate additional transcription factors, either positively or negatively, to fine-tune anthocyanin biosynthesis [40]. Protein–protein interactions within the complex are mediated by conserved domains, with bHLH proteins serving as molecular bridges between MYB and WD40 components [38]. The activity and stability of the MBW complex are further modulated by developmental cues and environmental factors, ensuring precise regulation of anthocyanin production

4. Anthocyanin biosynthesis activation is mediated by light

Light is among the most influential environmental cues regulating anthocyanin biosynthesis in plants. Through a suite of specialized photoreceptors, plants perceive distinct wavelengths of light and initiate transcriptional programs that modulate pigment accumulation accordingly.

UV-B radiation (280–315 nm) is particularly effective in inducing anthocyanin biosynthesis across angiosperms [41–43]. This response is mediated by the UV-B-specific photoreceptor UVR8 (UV RESISTANCE LOCUS 8), which undergoes monomerization upon UV-B exposure and translocates to the nucleus. There, UVR8 interacts with COP1 to regulate the expression of downstream genes involved in anthocyanin production [10]. In peach (*Prunus persica*), both UVA and UVB irradiation stimulate anthocyanin accumulation, with combined treatments producing additive effects [42]. Similarly, purple pepper (*Capsicum* spp.) exhibits robust anthocyanin induction under UV-B exposure [43]. In blueberries (*Vaccinium* spp.), UV-B treatment elicits stage-specific responses, suggesting that distinct regulatory mechanisms operate during fruit development and maturation [41].

Blue light also promotes anthocyanin biosynthesis, primarily through signaling pathways mediated by cryptochromes, which are photoreceptors that respond to blue wavelengths and activate transcriptional regulators of the biosynthetic pathway [10,39]. In red lettuce (*Lactuca sativa*), blue light significantly enhances anthocyanin content, although UV-B remains the more potent inducer [45]. In the purple tomato (mutant *Anthocyanin fruit*, *Aft*), combined exposure to blue light and UV-B yields synergistic effects, resulting in maximal anthocyanin accumulation [46].

Although less potent than UV and blue light, red light also contributes to anthocyanin biosynthesis through phytochrome-mediated signaling. Phytochromes perceive red and far-red wavelengths and integrate light signals to modulate pigment production [10,45]. The convergence of signals from multiple photoreceptors allows plants to finely adjust anthocyanin biosynthesis in response to complex light environments [47].

Consequently, anthocyanin biosynthesis is highly dependent on light availability. Under dark or low-light conditions, accumulation is significantly reduced, which negatively affects the commercial quality of horticultural crops. Some species that naturally accumulate anthocyanins when exposed to light develop a completely non-pigmented phenotype when the organ develops in dark conditions [8,9,12,48–51].

5. COP1 is an E3 ubiquitin ligase that regulates protein degradation

Post-translational regulation via the ubiquitin-proteasome system (UPS) is a central mechanism by which plants control protein turnover in response to developmental signals and environmental stress. This system enables selective degradation of proteins, allowing rapid cellular adaptation and reprogramming [11]. The specificity of the UPS is largely determined by E3 ubiquitin ligases, which constitute the most diverse class within the ubiquitination machinery and are responsible for recognizing and targeting substrates for degradation [52]. The UPS operates through a conserved three-step enzymatic cascade involving E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase). In this process, E1 activates ubiquitin in an ATP-dependent manner and transfers it to E2, forming an E2–ubiquitin conjugate. The E3 ligase then facilitates the transfer of ubiquitin from E2 to specific lysine residues on the target protein, marking it for degradation by the 26S proteasome [53] (Figure 2).

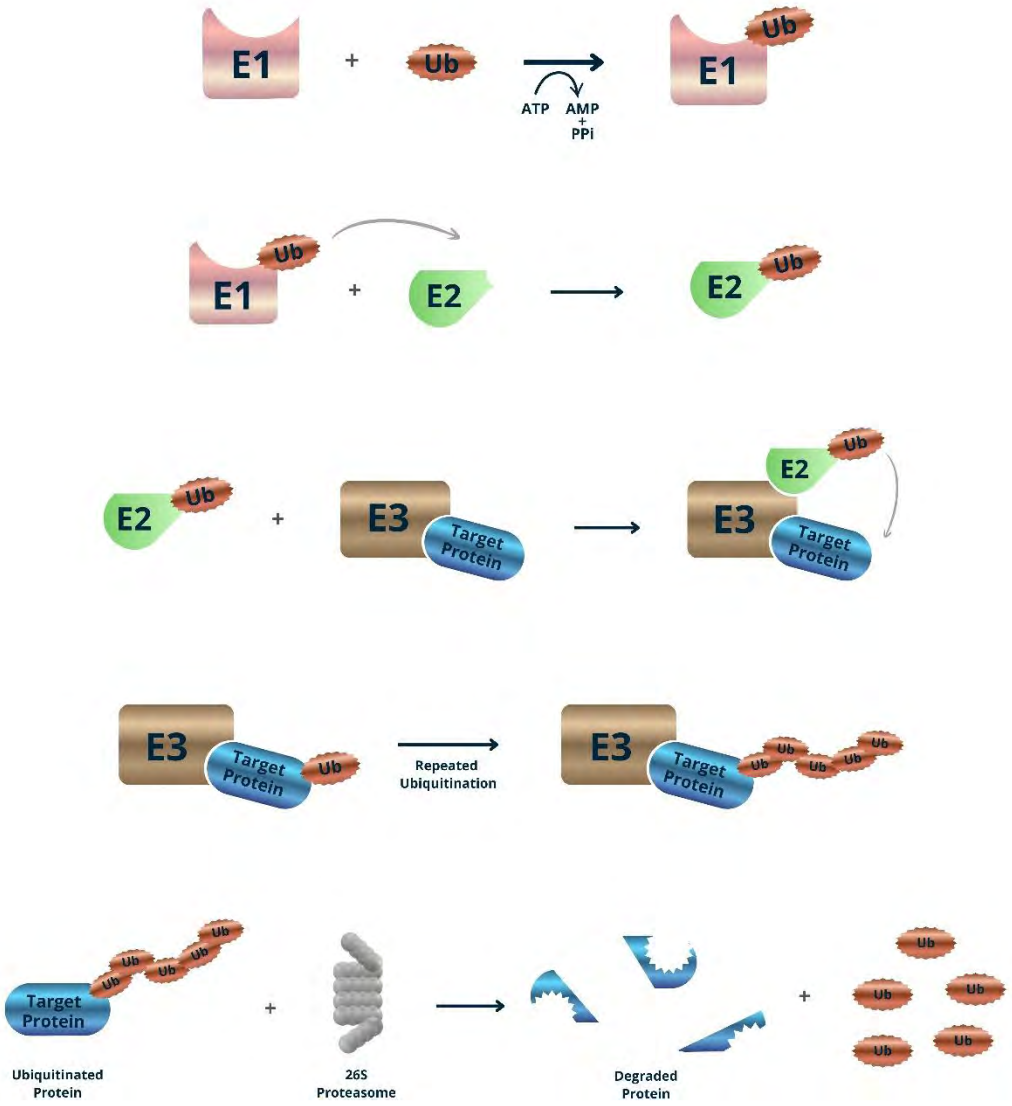

Figure 2. Post-translational regulation mediated by the ubiquitin–proteasome system. Ubiquitination occurs through a three-enzyme cascade that conjugates ubiquitin (Ub) to specific substrate proteins: the E1 ubiquitin-activating enzyme uses ATP to form a high-energy thioester with Ub, Ub is then transferred to an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase confers substrate specificity by binding both the E2-Ub complex and the target protein to catalyze Ub transfer to a lysine residue on the substrate. COP1 functions as an E3 ubiquitin ligase.

Plant E3 ligases are classified into four major families based on their structural domains and mechanisms of action: RING-type ligases, which contain a RING finger domain that promotes E2–E3 interaction; U-box ligases, which share functional similarities with RING-type ligases but possess distinct structural motifs; SCF complexes (SKP1–Cullin–F-box), which form multi-subunit assemblies for targeted degradation; and HECT-type ligases, which are less common but play essential roles in specific regulatory contexts [54,55]. These ligases are

involved in a wide array of physiological processes, including hormone signaling, stress response [11,56], development, light signaling [57], and immune regulation [58,59]. Among them, COP1 is a well-characterized RING-type E3 ligase that acts as a central repressor of light signaling pathways. By targeting key transcription factors for ubiquitination and subsequent degradation, COP1 plays a pivotal role in modulating photomorphogenic development and anthocyanin biosynthesis.

6. COP1 regulation of anthocyanin-related genes in different species

Anthocyanin biosynthesis in plants is tightly regulated by a conserved light-dependent signaling network, in which COP1 functions as a central E3 ubiquitin ligase. Under dark conditions, COP1 targets key transcription factors for ubiquitination and subsequent degradation via the 26S proteasome, thereby repressing pigment accumulation in response to environmental light cues. One of COP1's primary substrates is HY5, a bZIP transcription factor that integrates light signals and activates anthocyanin-related genes by binding to G-box elements in their promoters [10,42]. Consequently, COP1-mediated HY5 degradation in the dark suppresses anthocyanin biosynthesis [60–62]. However, the regulatory role of COP1 extends beyond HY5, as multiple studies have demonstrated its involvement in the degradation of other anthocyanin-related transcription factors, resulting in reduced pigment accumulation [12,14,49,50,63–67].

The first Arabidopsis *cop1* mutant, identified in the early 1990s, exhibits constitutive photomorphogenic development in the dark, characterized by short hypocotyls, open cotyledons, and light-responsive gene expression in the absence of light [68]. COP1 regulates a broad array of proteins, including HY5, HFR1, CONSTANS, PIFs, DELLA proteins, and notably PAP1 (MYB75) and PAP2 (MYB90), which are key regulators of anthocyanin biosynthesis within the MBW complex [14,69–72]. Loss-of-function mutants and RNAi lines targeting PAP1 exhibit reduced anthocyanin levels, while overexpression of PAP-related MYBs enhances pigment accumulation [73,74]. Importantly, PAP1 overexpression increases anthocyanin levels only in light-grown seedlings, with no comparable effect in the dark [75]. Despite similar transcript levels, PAP1 and PAP2 proteins accumulate more in light-grown seedlings, indicating post-translational regulation. Co-immunoprecipitation and proteasome inhibition assays confirmed that these MYB proteins interact with COP1 and are degraded in the dark, while light exposure stabilizes them, promoting anthocyanin biosynthesis [14].

A similar mechanism operates in apples (*Malus domestica*), where MdMYB1 is the principal R2R3-MYB transcription factor driving anthocyanin biosynthesis [76,77]. Although MdMYB1 transcript levels are higher in light-exposed tissues, protein accumulation is suppressed in the dark due to degradation mediated by MdCOP1-1 and MdCOP1-2, homologs of Arabidopsis COP1. These homologs physically interact with MdMYB1 and promote its ubiquitination and proteasomal degradation. Functional complementation of the Arabidopsis *cop1-4* mutant with MdCOP1s restored photomorphogenic traits, confirming their functional equivalence. Overexpression of MdCOP1s in apple fruit tissues reduced pigmentation, while silencing enhanced coloration, without altering MdMYB1 transcript levels [12], highlighting a conserved post-translational regulatory mechanism.

In eggplant (*Solanum melongena*), anthocyanin biosynthesis is strongly light-dependent, especially in cultivars such as ‘Lanshan Hexian’. Fruits grown in the dark develop a white phenotype lacking anthocyanins, while exposure to sunlight restores pigmentation. SmMYB1 and SmMYB5 are key transcription factors in this pathway and are targeted by SmCOP1 [49,50]. Under light conditions, SmMYB1 expression is upregulated, whereas SmCOP1 is more abundant in dark-grown fruit peel. Protein interaction studies confirmed that SmCOP1 promotes SmMYB1 degradation via the 26S proteasome, suppressing anthocyanin biosynthesis [50]. SmMYB5, although light-responsive and independent of SmHY5, also undergoes COP1-mediated degradation in the dark. MG132 treatment confirmed the interaction of SmCOP1 with SmMYB5, reinforcing the role of light in stabilizing anthocyanin-promoting proteins [49].

Further evidence from transgenic eggplant lines with RNAi-mediated silencing of SmCIP7, an ortholog of Arabidopsis *COP1-Interacting Protein 7* (*AtCIP7*), revealed reduced anthocyanin pigmentation accompanied by transcriptional reduction of the bHLH transcription factor *SmTT8* [63]. Although SmCIP7 does not directly interact with SmTT8, it may modulate COP1-mediated ubiquitination of transcription factors that regulate SmTT8, suggesting a positive regulatory role in anthocyanin biosynthesis.

In pear (*Pyrus* spp.), COP1 also acts as a negative regulator. In the ‘Red Zaosu’ variety of red pear (*P. pyrifolia*), fruits grown in the dark remain unpigmented, but exposure to blue light induces anthocyanin accumulation. Protein-protein interaction assays demonstrated that PpCOP1 interacts with PpMYB10, and two COP1-like genes, PbCOP1.1 and PbCOP1.2, were identified in Chinese pear, with expression inversely correlated with anthocyanin levels [64,66]. Overexpression of these genes suppressed pigmentation, while the bHLH transcription factor PpbHLH64, which interacts with PpMYB10 to activate *PpUFGT* expression, was shown to be

degraded in the dark via COP1-mediated proteolysis [65], indicating that bHLH proteins are also COP1 targets.

In tomato, anthocyanin biosynthesis involves a two-step MBW complex formation. SlJAF13, a bHLH transcription factor, forms the first MBW complex with SlAN2-like (MYB114/ANTHOCYNIN FRUIT) and SlAN11, activating *SlAN1* expression. SlAN1 then participates in the second MBW complex to activate structural genes [40]. Although SlJAF13 is constitutively expressed, its protein levels increase under light and decrease in the dark, suggesting post-translational regulation. SlCOP1 interacts with SlJAF13 and promotes its degradation via the proteasome, as confirmed by MG132 treatment and overexpression assays [67]. *SlCOP1* expression is elevated in non-pigmented fruit tissues and may regulate SlAN2-like protein stability, although further validation is required [48].

Across these five species, Arabidopsis, apple, eggplant, pear, and tomato, COP1 consistently functions as a negative regulator of anthocyanin biosynthesis by targeting key transcription factors for degradation. While the core mechanism is conserved, in which light inhibits COP1 activity and stabilizes anthocyanin-promoting proteins, species-specific differences emerge in the diversity of COP1 targets and the complexity of their regulatory networks. Arabidopsis COP1 regulates a broad spectrum of transcription factors, whereas apple and pear COP1s primarily target MYB and bHLH proteins. In eggplant, COP1 modulates multiple MYBs and SmCIP7, and in tomato, it targets bHLH components of the MBW complex. These findings underscore the evolutionary conservation of COP1-mediated post-translational control and its pivotal role in light-dependent pigmentation across diverse plant species.

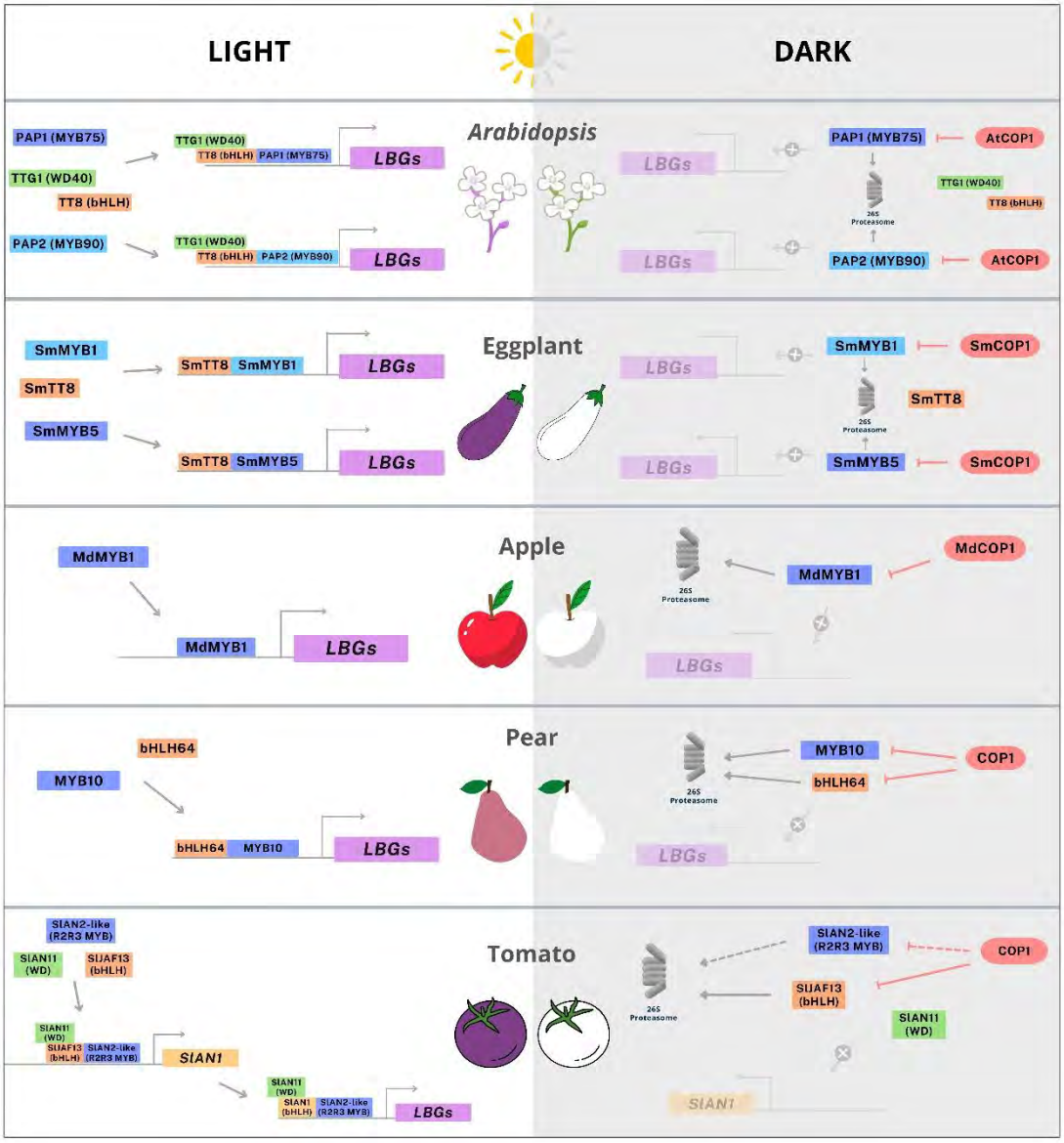

Figure 3. Summary of COP1 regulation via the 26S proteasome, affecting anthocyanin-related genes in Arabidopsis [14], eggplant [49,50], apple [12], pear [64,65], and tomato [47,67]. Gray arrows indicate activation; red lines indicate repression; dashed arrows and lines represent possible control mechanisms.

7. Conclusion

Recent advances have significantly deepened our understanding of light-regulated anthocyanin biosynthesis, particularly in relation to transcriptional control. However, post-translational mechanisms remain comparatively underexplored, despite their critical role in fine-tuning pigment accumulation. As highlighted throughout this review, COP1 functions as a conserved negative regulator of anthocyanin biosynthesis under dark conditions across multiple plant species. Its activity is not restricted to a single class of transcription factors; rather, COP1

targets a diverse array of regulatory proteins, including MYB and bHLH components of the MBW complex, as well as other modulators of pigment biosynthesis.

This evidence underscores the importance of post-translational regulation as a complementary layer to transcriptional control in the anthocyanin biosynthetic pathway. A comprehensive understanding of anthocyanin regulation requires integration of both transcriptional and post-translational networks, particularly given the dynamic nature of environmental responses and developmental cues. Such integrative knowledge holds substantial promise for crop improvement strategies. By manipulating key regulatory nodes, both at the gene expression and protein stability levels, it may be possible to engineer anthocyanin-enriched cultivars with enhanced stress resistance, improved nutritional profiles, and greater visual appeal. Future research should prioritize the elucidation of these regulatory interactions to enable precise and sustainable modulation of anthocyanin traits in economically important crops**.**

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Abbreviations

The following abbreviations are used in this manuscript:

4CL	4-coumarate:CoA ligase
AN1	ANTHOCYANIN 1 protein (bHLH transcription factor homologous to TT8)
AN11	ANTHOCYANIN 11 protein (WD40 protein, component of the MBW complex)
AN2	ANTHOCYANIN 2 protein (MYB transcription factor homologous to PAP1/MYB75)

ANS	anthocyanidin synthase
ANS	anthocyanidin synthase (also called LDOX, leucoanthocyanidin dioxygenase)
bHLH	basic helix–loop–helix transcription factor family
C4H	cinnamate 4-hydroxylase
CHI	chalcone isomerase
CHS	chalcone synthase
CIP	COP1-interacting protein (generic term; also used for specific COP1 partners in some studies)
CONSTANS	CONSTANS (CO; photoperiodic flowering regulator, B-box zinc-finger TF)
COP1	CONSTITUTIVE PHOTOMORPHOGENIC 1
DELLA	DELLA proteins (GA signaling repressors; named from conserved DELLA motif)
DFR	dihydroflavonol 4-reductase
E1	ubiquitin-activating enzyme
E2	ubiquitin-conjugating enzyme
E3	ubiquitin ligase
EBGs	early biosynthetic genes
F3'5'H	flavonoid 3',5'-hydroxylase
F3'H	flavonoid 3'-hydroxylase
F3H	flavanone 3-hydroxylase
GST	glutathione S-transferase (anthocyanin-binding/transport-associated GSTs)
HECT	Homologous to the E6-AP Carboxyl Terminus (E3 ubiquitin ligase domain)
HFR1	LONG HYPOCOTYL IN FAR-RED 1 (bHLH transcription factor)
HY5	ELONGATED HYPOCOTYL 5
JAF13	Jasmonate-associated factor 13 (bHLH transcription factor)
LBGs	late biosynthetic genes
MBW	MYB–bHLH–WD ternary regulatory complex
MG132	carbobenzoxy-Leu-Leu-Leu-al (proteasome inhibitor)
MYB	Myeloblastosis (MYB) DNA-binding transcription factor family
PAL	phenylalanine ammonia-lyase
PAP	PRODUCTION OF ANTHOCYANIN PIGMENT (e.g., PAP1/MYB75; PAP2/MYB90 in Arabidopsis)
PAT	putative anthocyanin transporter

PIFs PHYTOCHROME-INTERACTING FACTORS (bHLH transcription factor family)
RING Really Interesting New Gene (RING) zinc-finger domain (common E3-ligase type)
RNAi RNA interference
ROS reactive oxygen species
SCF SKP1–Cullin–F-box E3 ubiquitin ligase complex
TT8 TRANSPARENT TESTA 8 (bHLH transcription factor regulating flavonoid/anthocyanin genes)
UFGT UDP-glucose:flavonoid 3-O-glucosyltransferase
UPS ubiquitin-proteasome system
UVR8 UV RESISTANCE LOCUS 8
WD40 WD40 repeat protein (often a scaffold protein; e.g., TTG1)

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THIRD PART

5 FINAL CONSIDERATIONS

This thesis advances the understanding of anthocyanin biosynthesis in tomato by integrating transcriptional and post-translational regulatory mechanisms. Through two complementary studies, it highlights both the environmental cues and molecular players that govern pigment accumulation, thereby offering new insights into strategies for nutritional biofortification.

The first article demonstrated that anthocyanin accumulation in purple tomato fruits is strongly dependent on light penetration, with pigmentation restricted to the peel due to the shielding effect of the cyanic epicarp. Transcriptional analyses identified *SlAN1* as a limiting factor for light-dependent anthocyanin activation, underscoring the importance of tissue-specific regulation. These findings reveal how anatomical and environmental factors interact to shape pigment distribution, providing a framework for targeted breeding approaches.

The second article synthesized recent advances in COP1-centered regulation of anthocyanin biosynthesis, emphasizing its role as a post-translational repressor that extends beyond HY5. Evidence from multiple horticultural species, including tomato, suggests that COP1 interacts with diverse transcription factors and regulatory modules, thereby orchestrating pigment repression under dark conditions. This broader perspective situates COP1 as a central hub in anthocyanin regulation, opening opportunities to manipulate its activity for enhanced pigment accumulation.

Together, these studies converge on a central theme, in which anthocyanin biosynthesis in tomato is controlled by a dynamic interplay between light accessibility, transcriptional activators, and post-translational repressors. By dissecting these mechanisms, this thesis contributes to a more comprehensive understanding of how pigmentation is coordinated across fruit tissues. Importantly, it establishes a conceptual foundation for future research aimed at overcoming the paradox of light-dependent regulation in pigmented tissues and at utilizing COP1's regulatory network for crop improvement.

Beyond plant physiology, the implications of this work extend to human health. Anthocyanins are valued for their antioxidant and anti-inflammatory properties, yet the optimal dietary thresholds for measurable benefits remain undefined. By linking tissue-specific accumulation patterns with nutritional outcomes, this research supports the development of functional foods enriched in both peel and flesh, thereby maximizing dietary impact.

In conclusion, this thesis underscores the dual importance of transcriptional and post-translational regulation in anthocyanin biosynthesis. It highlights *SlAN1* and COP1 as pivotal regulators whose manipulation could enable the creation of tomato cultivars with uniformly high anthocyanin content. Such advances would not only enhance the nutritional value of one of the world's most consumed vegetables but also provide a model for biofortification strategies in other horticultural crops. Ultimately, the integration of molecular genetics, light biology, and nutritional science paves the way for innovative approaches to improve human health through plant-based diets.

Perspectives on the regulation of anthocyanin biosynthesis in tomato and beyond

Based on our recent findings about the influence of light on anthocyanin biosynthesis in purple tomatoes, a promising research avenue emerges that integrates both transcriptional and post-translational mechanisms. The potential influence of short wavelengths, such as blue and UV-B light, activating regulatory and structural anthocyanin genes in the epicarp, but are blocked by early pigmentation, suggests a fascinating biological paradox. To overcome this limitation, one experimental direction would be the development of genetic lines with temporary suppression of epidermal pigmentation, thereby allowing deeper light penetration into inner fruit tissues. This strategy could be implemented using gene editing tools (e.g., CRISPR-Cas9) to transiently silence early pigment synthesis genes such as *SlAN1*, followed by evaluation of anthocyanin accumulation in the mesocarp. Such an approach would clarify whether light accessibility alone is sufficient to trigger pigment biosynthesis in inner tissues.

In parallel, the role of COP1 as a post-translational regulator that represses anthocyanin biosynthesis under dark conditions raises important questions about its broader interactions with transcription factors beyond HY5, including MYB and bHLH proteins. Future experiments should focus on generating tomato mutants with reduced or null expression of *COP1 homolog* (*Solyc12g005950*), assessing whether this modulation enables anthocyanin pathway activation even under low-light environments. Complementary proteomic studies could map COP1's specific targets within the MBW complex, revealing new regulatory layers that extend beyond transcriptional control. This would provide a more comprehensive understanding of how COP1 coordinates pigment repression across multiple substrates.

Another critical perspective involves the integration of epidermal pigmentation control with manipulation of post-translational regulators. Combining these approaches could enable the development of tomato cultivars with high anthocyanin content distributed throughout the entire fruit, rather than being restricted to the peel. Such cultivars would not only enhance

nutritional value but also increase commercial appeal. To validate these strategies, field trials under varying light regimes, coupled with transcriptomic and proteomic analyses, will be essential to assess stability, yield, and pigment distribution under real-world conditions.

Beyond plant physiology, future research should also address the human health dimension of anthocyanin biofortification. The optimal dietary intake of anthocyanins required to confer measurable health benefits remains poorly defined. Studies that establish these thresholds, while correlating them with tissue-specific accumulation patterns in fruits and vegetables, would be highly valuable. A comparative approach examining the health benefits derived from epicarp (high pigmentation) versus mesocarp (lower pigmentation) could justify the development of functional foods enriched in both tissues. This would support the design of tomato cultivars capable of delivering optimal anthocyanin levels throughout the fruit, ensuring maximum nutritional impact.

Finally, given the decisive roles of SlAN1 and COP1 in regulating anthocyanin accumulation across different tomato fruit tissues, functional validation studies are desirable. Developing mutants that combine loss-of-function and overexpression of *SlAN1* with similar manipulations of *COP1 homolog* will provide critical insights into their synergistic or antagonistic roles in the anthocyanin pathway. Such experiments will help unravel the relative contributions of transcriptional activation versus post-translational repression, ultimately guiding targeted breeding and biotechnological strategies for anthocyanin enrichment.